UNIVERSITY OF EDINBURGH

Bacteriological Studies on Johne's Disease in Cattle and Sheep

Thesis presented for the Degree of Doctor of Science

by

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Much of the fundamental knowledge of Johne's disease was provided in this country by Twort and Ingram, who first succeeded in cultivating the causal organism, and by McFadyean and his co-workers in the years prior to 1920. During the succeeding years the disease has presented a problem of gradually increasing importance, a process which has recently been greatly accelerated by the development of means to control or greatly to diminish, if not always to eradicate, the major infectious diseases of cattle in this country. Of these so-called major diseases, namely tuberculosis, contagious abortion and streptococcal mastitis, Johne's Disease alone still provides a serious problem to veterinary science and to agriculture.

The work described in this thesis was undertaken over a period of rather more than ten years, first at the Animal Diseases Research Association, Moredun Institute, Edinburgh, and later at the Agricultural Research Council Field Station, Compton, Berkshire, and the papers, although not in chronological order, may conveniently be presented in three parts.

I.

A study of Johne's disease in sheep, consisting of four papers:-

"Ovine paratuberculosis (Johne's disease of sheep)".

(Thesis - page 6) J. comp. Path. 55. 41 1945

"Observations on the isolation of Mycobacterium johnei in primary culture".

(Thesis - page 13) J. Path. Bact. 62. 647. 1950

"Varieties of Mycobacterium johnei isolated from sheep".

(Thesis - page 21) J. Path. Bat. 63. 333. 1951

"The experimental infection of cattle with varieties of Mycobacterium johnei isolated from sheep".

(Thesis - page 29) In press

These papers have provided a new conception of the bacteriology of Johne's disease as it affects sheep. The disease has been shown to be caused in this country by one of two distinct organisms, the classical type of M. johnei and a highly pigmented organism, apparently a variant of the classical type. This variant, with the organism causing Johne's disease in sheep in Iceland, was originally regarded as uncultivable, but by greatly increasing the percentage of egg yolk in the medium used, both were regularly obtained in artificial culture. The fact that these acid-alcohol fast organisms are the cause of a disease in sheep simulating Johne's disease in cattle and that they require an accessory factor in the form of killed acid fast bacilli in the medium on which they are grown would indicate that they are variants of the classical type. This hypothesis has been shown to be correct by the reproduction of the syndrome of Johne's disease in cattle artificially infected with these organisms. On the other hand, their variation from the classical type has been shown to be "fixed" by the retention of their peculiar cultural requirements and in the case of the pigmented type its distinct colouration, after one passage through the bovine.

II.

Studies on the experimental disease in cattle and on the incidence of infection with M. johnei in cattle; three papers:-

"Experimental Johne's disease in cattle."
(Thesis - page 37) In press

"Observations on the incidence of infection with M. johnei in cattle."
(Thesis - page 53) Vet. Rec. 61. 539. 1949

"Further observations on the incidence of infection with M. johnei in cattle."
(Thesis - page 58) Vet. Rec. 64. 603. 1952

and a review

"Johne's disease - Its diagnosis and control."

(Thesis - page 65) Presented to the 69th Annual Congress of the National Veterinary Medical Association, 1951.

Vet. Rec. 63. 776. 1951

The first of these papers recorded a reliable method of reproducing Johne's disease in cattle, a fundamental requirement for serious research but one which had been somewhat neglected in the past and several previously unrecognised facts regarding the disease. Thus, animals infected with M. johnei in early life may excrete the organism in their faeces for many months before clinical signs become apparent, and on the other hand, cattle which during life appear in normal health and at autopsy show no evidence of the disease may yet harbour the organism in the intestine or mesenteric lymph nodes. Further, it had been thought in the past that in the affected animal M. johnei was confined to the intestine and to its associated lymph nodes, but it has now been shown both in experimentally induced and natural clinical cases of Johne's disease that the organism may also be recovered in culture from a variety of the other tissues and lymph nodes.

Johne's disease is known to occur among cattle throughout Great Britain, but there are no figures available regarding the actual incidence of the disease. The second and third papers, however, record that the cultural examination of a single mesenteric lymph node from each of 665 apparently normal cattle slaughtered for human consumption in Berkshire yielded M. johnei 101 times, or from 15 per cent. It is unlikely that all of these infected animals would have eventually developed the clinical disease, but this very high infection rate among what may be regarded as healthy cattle may explain, in part at least, the occurrence of fresh outbreaks

outbreaks of the disease, and the very high incidence of "non specific sensitivity" to mammalian tuberculin which occurs in many parts of the country.

"Johne's disease - its diagnosis and Control," is a review of some aspects of the literature on the disease which have been published since 1933; it discusses much of the work described in the other papers of this thesis.

III.

Two papers written in collaboration:-

"Observations on the blood picture of Johne's disease in sheep and cattle with special reference to the magnesium content of the blood."

with James Stewart and Jennie W. McCallum.

(Thesis - page 81) J. Comp. Path. 55. 45. 1945

This work showed that in sheep and cows exhibiting clinical symptoms of Johne's disease the magnesium content of the blood may be very low, and it was suggested that this lowering of the blood magnesium might become a useful aid in diagnosis. However, since a low blood magnesium may have a variety of causes, the practical value of this test has not been established.

"The filtration of Mycobacterium tuberculosis and Mycobacterium stercusis through gradocol membranes."

with M.A.Soltys

(Thesis - page 87) J. Path. Bact. 56. 173 1944

The possibility of the existence of a form of M. tuberculosis or other Mycobacterium capable of passing the pores of bacteria proof filters and of giving rise to disease on inoculation into susceptible animals was a

a source of controversy for many years. The subject was extensively reviewed in the 2nd edition of Topley and Wilson's "Principles of Bacteriology and Immunity," and the conclusion reached that no satisfactory evidence had been brought forward to support the existence of such forms. In the 3rd edition they state, "The more recent findings of Soltys and Taylor support this conclusion" and the review of the subject has been omitted.

Ovine Paratuberculosis (Johne's Disease of Sheep).

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INTRODUCTION

Johne's disease has long been known to be transmissible to sheep, and naturally occurring cases of the disease in these animals have been reported from Europe, India and North America.

McFadyean (1916), and later, Dunkin (1934), suggested that the disease might be more widespread in this country than was realised and Wilson (1934) has recorded the comparative frequency with which Johne's disease in sheep may be encountered in Scotland.

The earlier workers, Twort and Ingram (1913) and McFadyean (1916), in this country, and Howarth (1932) in North America, experienced no difficulty in cultivating the causal organism on suitable media, but more recently Dunkin and Balfour-Jones (1935) found that it grew in primary culture only after a long period of incubation while McEwen (1939), using a variety of different media, was unable to obtain growth even after incubation for periods up to 12 months.

It is the purpose of this paper to confirm the implication in the work of previous authors that there are two closely related organisms responsible for Johne's disease of sheep.

PRESENT INVESTIGATION

Within recent years Johne's disease has been diagnosed at Moredun Institute in sheep from most of the border counties of Scotland and also from the Lothians, Lanark and Sutherland. Many of these animals came from hill flocks, and in no instance was there a history of the sheep having been associated with cattle affected with chronic enteritis. Commonly the disease would appear to be sporadic in its occurrence, only one or two cases appearing among a flock, but on other farms several "pining"

"pining" animals may be lost every year. Only rarely have cases been seen in animals under the age of three years, the majority occurring in ewes of three years and over and in these animals clinical symptoms are usually first observed in late winter and spring.

The clinical signs are those of a severe debilitating disease. The animal loses condition, the wool is loosened, emaciation develops and death generally supervenes within two to three months. Diarrhoea is a remarkably inconstant symptom and may not be observed although often present intermittently. Diagnosis during life may not be easy; numerous acid-alcohol fast organisms conforming to typical M. johnei may be present in the faeces, but the number may not always be sufficiently large to warrant a definite diagnosis, and the results obtained with avian tuberculin and with johnin are of doubtful value. As is shown elsewhere (Stewart, McCallum and Taylor, 1944), the biochemical examination of blood and the demonstration of a fall, in blood magnesium may be of considerable assistance in establishing a diagnosis.

At autopsy, the carcase of an animal which has shown clinical symptoms of the disease is generally emaciated and there may be an almost complete absence of body fat; otherwise the lesions are confined to the intestine and mesenteric lymph nodes which are oedematous and may be very much enlarged. Extensive lesions in the bowel have been found, however, on two occasions even in animals in very good bodily condition, slaughtered for other purposes. Lesions in the intestine may be confined to the posterior part of the ileum, but nearly the whole of the small and large bowel may be affected.

Macroscopically, the lesion consists of a thickening of the mucous coat, but in some cases this may be so slight as to be almost unnoticeable. Corrugations such as are seen in the bovine disease may or may not be present

present, but are never so prominent as in cattle. As was described by Wilson (1934) the mucous surface of the affected bowel in the majority of cases is stained yellow with bile and in some animals may even assume a deep orange colour.

Microscopically, smear preparations from the affected bowel usually show enormous numbers of acid-alcohol fast organisms and may even present the appearance of a culture film containing a few cells. The pathological histology of Johne's disease in cattle is well known and in sheep has been described at some length by McEwen (1939), who confirmed the opinion of earlier workers that the lesions were essentially similar to those found in cattle.

Cultivation of the Organism

At Moredun Institute, prior to 1939, numerous attempts to cultivate the acid-alcohol fast organisms associated with the lesions of Johne's disease in sheep had proved entirely negative. Since that date different media have been inoculated with material from 15 affected sheep from eleven farms. From four of these animals, all from the same farm, an organism apparently identical with M. johnei (M. paratuberculosis, Johne's bacillus) was isolated without difficulty, but from the remainder growth was either not obtained or was very slight.

Materials and Methods:- The most suitable medium has been found to be that described by Minett (1942) containing whole egg and liver extract with 4 per cent. of glycerine, 1 per cent. of heat killed M. phlei, and a suitable concentration of gentian violet. Latterly, the dye has been omitted, and it has been found advisable to autoclave the phlei suspension before incorporating it in the medium in order to ensure the death of the organisms.

Numerous modifications of this medium have been tried. Egg yolk has been used instead of whole egg with or without the addition of liver extract; serum and/or potato extract have been added, the proportion of glycerine has been varied and tuberculin or finely minced affected sheep bowel has been employed in place of M. phlei. The media were distributed and inspissated in screw-cap bottles or test tubes which after inoculation were sealed with paraffin wax or rubber caps.

Infected material has been treated with antiformin or 5 per cent. oxalic acid prior to inoculation or in some cases inoculated direct after aseptic dissection through the peritoneal surface of the bowel. Lymph node has also been inoculated direct. The cultures have been incubated at 37°C. to 38°C. for periods up to two years.

In order to control the suitability of the media for M. johnei, several bovine strains were isolated and maintained throughout the investigation.

Results - Of the 15 cases from which media were inoculated a normal growth of M. johnei was obtained from four. These four cases all came from the same farm and presented features at autopsy which varied somewhat from the remainder. The affected bowel in each was greatly thickened but without marked pigmentation. and in three instances organisms were only found with difficulty. Films from the intestine of the fourth sheep showed bacilli which, although numerous, were not in such numbers as are usually associated with the ovine disease. Media inoculated from these animals showed growth which was visible to the naked eye after four weeks' incubation and at the end of eight weeks was almost profuse. The primary cultures were subcultivated on to plain egg, glycerine egg and glycerine agar as well as on to a phlei medium. Subcultures grew only on the last, and it was therefore assumed that the organism was of the classical M. johnei type.

In the remaining cases, growth either did not occur or was observed as an increase in the number of organisms present in films, and even this evidence of multiplication was not demonstrable until after incubation for from 12 to 16 months. Subcultivation at this stage did not lead to an acceleration of growth but merely to a further increase in the number of bacilli present in films after a similar period of incubation. Prolongation of incubation up to periods of 24 months gave no further increase. At no stage was the growth definitely visible to the naked eye.

DISCUSSION

It is apparent from the somewhat conflicting reports of other authors and the work described in this paper that Johne's disease in sheep may be caused by one of two closely allied but different organisms. The first of these conforming to the classical M. johnei has long been recognised as a pathogen of cattle and sheep. The second organism, which as suggested by Taylor (1940) should be distinguished as the ovine type, is the cause of an essentially similar disease and may only be differentiated from the first by its resistance to artificial cultivation. It may show a tendency to multiply slightly on artificial media after incubation for at least one year, but apart from one strain isolated by Dunkin and Balfour-Jones (1935), satisfactory growth has never been obtained. The very close relationship between this organism and M. johnei was demonstrated by McEwen (1939), who succeeded by its use in artificially infecting cattle which eventually developed symptoms and at autopsy, characteristic lesions of Johne's disease.

Paratuberculosis would appear to be a disease of comparatively low morbidity. Admittedly most of the cases examined came from hill flocks where the chances of infection must be considerably less than on enclosed

enclosed land, but the statement is based on the fact that many clinical cases occur sporadically and only occasionally can a history of chronic emaciation among the stock be obtained. It has been shown that the disease is widespread in Scotland and there can be little doubt that its presence may often be unsuspected, particularly as many cases can be diagnosed only by a microscopic examination of stained films and that lesions may be present in the absence of clinical symptoms.

SUMMARY

Paratuberculosis or Johne's disease of sheep would appear to be widespread in Scotland.

The disease may be caused by either of two organisms, the classical type of M. johnei and a closely related organism which is only differentiated by its resistance to artificial cultivation and which may be termed M. johnei (ovine type).

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Observations on the isolation of *Mycobacterium johnei* in primary culture

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The cultivation of Mycobacterium johnei from bovine tissue, or more especially from faeces, is complicated in that its habitat is often heavily contaminated by other organisms, it requires an accessory factor in the form of killed M. phlei or tuberculin in the medium, and visible growth need not be expected until at least the fourth week of incubation; moreover, it is non-pathogenic for laboratory animals. Minett (1942) reviewed the media and methods which have been recommended for the isolation of M. johnei and suggested that the best results were obtained by the preliminary treatment of the material with antiformin followed by inoculation on Dunkin's medium (Dunkin, 1928), which is an inspissated mixture of whole egg and liver extract with 4 per cent. of glycerine and about 1 per cent. of heat-killed M. phlei; he found this medium superior to those with a basis of egg-yolk or solid serum, or to potato medium.

This paper describes the result of attempts to improve and simplify both the methods of isolation and the media used in the primary cultivation of M. johnei.

Preliminary treatment of material

1. Tissue: A simplification of the oxalic-acid method devised by Corper and Uyei (1929-30) has proved by far the most satisfactory way of destroying contaminants in tissue. The material is ground in a mortar with sand and a 5 per cent. w/v solution of oxalic acid in distilled water, in the proportion of roughly 1 g. of tissue to 10 ml. of oxalic acid. The suspension is filtered through four layers of butter muslin, incubated for thirty minutes at 37°C. in a water-bath and spun for 20-30 minutes at approximately 3500 r.p.m. The sediment, without further treatment, is then sown thickly on at least five McCartney bottles of solid medium. This method has been successfully used over a number of years with lymph-node, liver, lung, spleen and intestine,

intestine, both fresh and in a semi-decomposed condition. Antiformin, caustic soda and sulphuric acid are not only more laborious to use, as they require neutralisation or washing out of the final inoculum, but do not give such satisfactory results. Tribasic sodium phosphate (Corper and Stoner, 1946), said to be non-toxic to tubercle bacilli, has similar disadvantages.

2. Faeces: There is at present no completely reliable method of obtaining M. johnei in culture from infected bovine faeces, particularly from samples in which they may be present in very small numbers. Treatment with antiformin remains the method of choice, as no other agent so far available is capable of destroying the contaminating organisms and spores, and of permitting subsequent growth of Johne's bacillus. This method is far from ideal, as it is more lethal to M. johnei than oxalic acid and considerable care and time are required in its use. None of the other methods commonly used in the isolation of M. tuberculosis are of value (Levi, 1948), even after preliminary suspension of the material overnight in a solution containing 100 units of penicillin per ml. Treatment of the faeces with a saturated solution of tribasic sodium phosphate ($Na_3PO_4 12 H_2O$: B.D.H.) for from five to seven days at 37°C. is sometimes effective, but is generally unreliable in that contamination is frequent and colonies are slow to develop.

The selection of medium

Liver-agar was at one time in almost universal use for the cultivation of the Brucella group, but it was rarely completely reliable, as it showed considerable batch variation and has been largely superseded by Bacto-tryptose agar. Dunkin's medium for M. johnei shows a similar defect in that one batch may give excellent growth, while another, prepared with a different

different brew of liver extract, will barely support growth. Further, it is increasingly well recognised (Corper and Cohn, 1933; Schwabacher, 1936-37; Finlayson, 1946) that in the cultivation of tubercle bacilli on egg media the essential constituent is yolk, and that complex media with a variety of ingredients are of no greater value than a simple medium with egg-yolk as a base; also the elimination of variable ingredients tends to standardise a medium so that it shows little or no variation from one batch to another.

Accordingly the following simple media, among others, were prepared and tested repeatedly, using Dunkin's medium contrast-stained with Congo red instead of crystal violet as a control in every case:- Corper's egg-yolk (Corper and Cohn, 1933), the American Trudeau Society's egg-yolk potato medium (Committee on Evaluation of Laboratory Procedures, Report, 1946), Finlayson's egg medium (Finlayson, 1946), Herrold's egg-yolk agar (Herrold, 1931) and Jamieson's modification (Jamieson, 1938): Johnson's lymph-node medium (Johnson, 1944) and Dubos's medium (Dubos and Middlebrook, 1947).

Technical methods

To each medium, 4 per cent. of glycerine and roughly 1 per cent. w/v of heat-killed M. phlei are added. It has been found, with Twort and Ingram (1913) and Minett (1942), that more and larger colonies develop with 4 per cent. glycerine than with 0, 1, 2 or 6 per cent. Better results have been obtained with heat-killed M. phlei than by the addition of phlei-free culture-filtrates or extracts of the bacilli or with tuberculin. The culture of M. phlei is grown on the surface of glycerine broth for two to three weeks, when it is steamed. The organisms are removed by filtration and ground in a modified Griffith tube with glycerine and normal saline to give an even suspension which is then autoclaved to ensure the complete sterilisation of the organisms before addition to the medium.

Egg media are prepared with the usual aseptic precautions, dispensed in 1-oz. round screw-capped bottles and inspissated in the autoclave. The bottles are laid flat in the cold autoclave, the valves closed and heat applied. When the gauge shows a pressure of 15 lb. (approximately 90°C.), the heat is withdrawn and the autoclave allowed to cool before opening. The bottles are incubated for 3-5 days at 37°C. and those showing contamination are discarded. With reasonable attention to detail this method has proved highly satisfactory in that contaminated bottles are few in number; and both prolonged heating and fat on the surface of the medium, either of which will impair or inhibit growth (Corper and Clark, 1946; Cummings et al., 1948) are avoided.

Most of the media tested have been stained pink by the addition of 1 per cent. of a 1 per cent. solution of Congo red (Gurr). The dye at this concentration has no inhibitory effect upon the growth of micro-organisms and is included simply to give contrast, as young colonies of mycobacteria are not readily seen on unstained media containing a high proportion of egg-yolk, particularly in screw-capped bottles.

The inocula consist of both lightly and heavily infected bovine intestine and lymph-nodes after treatment with oxalic acid, infected bovine faeces treated with antiformin, and occasionally serial dilutions in Ringer of suspensions of M. johnei, using either the primary culture or the first subculture. Although there are objections to the use of natural material in comparative tests of media, it is inadvisable to use laboratory cultures of M. johnei for this purpose as the organism becomes less selective in its growth requirements on subcultivation.

RESULTS

The addition of blood charcoal to egg media was found by Nassau (1942)

(1942) to increase the rate of growth of human tubercle bacilli; no such effect was demonstrable with M. johnei. Corper's yolk medium is simple to prepare and consistent in quality, it gives a good growth of Johne's bacillus but has the marked disadvantage of being extremely soft in use. The American Trudeau Society's egg-yolk potato medium also gives good growth, but contains potato extract and may vary somewhat from batch to batch, depending probably upon the age and quality of the potatoes used. Both Herrold's and Jamieson's egg-yolk agar are somewhat awkward to prepare, and since they contain raw egg yolk are liable to contamination, but both will give a good if rather slow growth of M. johnei. Johnson's lymph-node medium, a modification of Dorset and Henley's synthetic medium with bovine lymph-node added, was devised for the cultivation of laboratory strains of M. johnei and is of little value for the isolation of primary cultures. Several attempts were made to obtain growth on Dubos's agar medium without success; but Davis and Dubos (1948) have recently explained that failure to isolate M. johnei on this medium is to be expected. Finlayson's medium containing 60 per cent. by volume of egg-yolk with 15 per cent of egg-white to improve the texture gives as good a growth of M. johnei as Corper's or the American Trudeau Society's medium, and is easy to prepare, has a firm surface and is remarkably consistent from batch to batch.

Of these media, those with the highest proportion of egg-yolk, namely Corper's (80 per cent.), Finlayson's (60 per cent.) and the American Trudeau Society's medium (50 per cent.) give somewhat similar results: all three are superior to Dunkin's medium. Finlayson's is preferred for the reasons given above and has been in routine use for the isolation of M. johnei in this laboratory for the last three years. The formula used is as follows:-

Egg-yolk	60 volumes
Egg-white	15 "
Glycerine	4 "
Congo red solution (1 per cent.)	1 "
Heat-killed M. phlei	1 "
Normal saline	19 "

This medium is reliable for the primary cultivation of M. johnei. For this purpose it is definitely superior to the widely used Dunkin's medium and permits the isolation of varieties of Johne's bacillus which do not grow on Dunkin's medium (paper in preparation). Of the several hundred batches which have been prepared in this laboratory, the only difficulties which have arisen have been due to overheating, with consequent extrusion of fat, and on one occasion to contamination of the phlei suspension.

SUMMARY

For the primary cultivation of Mycobacterium johnei from tissue, the use of oxalic acid is recommended; from faeces, antiformin is the only substance so far available which is effective in controlling contamination.

Simple media containing at least 50 per cent. of egg-yolk are more reliable than complex mixtures of whole egg and liver extract.

Acknowledgement is due to Mr. F. Summerfield, senior technician at this Field Station, for his careful supervision of the preparation of many different media.

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Varieties of Mycobacterium johnei isolated from sheep

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Taylor (1945) confirmed the implication in the work of earlier authors that there are two diseases of sheep simulating Johne's disease (paratuberculosis) of cattle. The first of these, true Johne's disease of sheep, has been recognised for many years and is known in Europe, India, and America; it is caused by the classical type of Myco. johnei, identical with the organism isolated from cattle. The second condition is widespread in Scotland and is characterised by the yellow or orange colour of the affected intestine and the enormous number of acid-alcohol fast bacilli present in the lesions. Attempts to isolate the organism responsible for this form of the disease have consistenly failed and cultures have not so far been described. Johne's disease of sheep in Iceland is another example of a similar condition caused by an organism resistant to artificial cultivation, for although the disease has been the subject of several recent papers (Sigurdsson and Tryggvadottir, 1949) cultures have never been obtained (Palsson, personal communication; Glover personal communication).

This paper describes the isolation in culture of strains of acid-alcohol fast organisms resembling Myco. johnei from sheep in Scotland and Iceland.

Materials and Methods

From 1948-50, fifteen specimens of intestine from different sheep affected with Johne's disease were received. One from Edinburgh was of the non-pigmented type; four with no unusual colouring came from Iceland; and ten showing the orange-coloured type of lesion came from Aberdeen and the north of Scotland.

Mucous membrane from each piece of intestine was treated with oxalic acid to destroy contamination before inoculation on a number of

of different media, which included some of the following: Dunkin's medium for Myco. johnei but without crystal violet; the American Trudeau Society's egg-yolk potato medium; a slightly modified Finlayson medium (Taylor, 1950) and Jamieson's egg-yolk agar. All of these contained 4 per cent. of glycerine and approximately 1 per cent. of heat-killed Myco. phlei. In addition, Finlayson's medium both with and without 4 per cent. of glycerine was used.

Results

The classical bovine type of Myco. johnei was isolated from one of the fifteen specimens examined: that received from Edinburgh. In primary culture it grew readily after four weeks' incubation on Dunkin's medium, modified Finlayson medium and the American Trudeau Society's medium. Culturally and microscopically the growth was typical of Myco. johnei. There was no growth on media without killed Myco. phlei.

Each of the four specimens from Iceland yielded a culture morphologically similar to Myco. johnei, but slower to develop. In primary culture no growth was apparent until the seventh week of incubation. It appeared on modified Finlayson's medium as very small whitish colonies which did not increase greatly in size on further incubation. Growth never resulted on Dunkin's medium either in primary culture or on first subculture, and profuse growth was not obtained on any medium even after the sixth subculture. Media without killed Myco. phlei do not support growth.

From each of the ten specimens of the orange-coloured type of lesion sent from the north of Scotland, highly pigmented strongly acid-alcohol fast bacilli were isolated. These grew well in primary culture on modified Finlayson medium after incubation for from 6 - 8 weeks and nearly as well on good batches of American Trudeau Society's medium; but growth

growth was not obtained on Dunkin's medium or Jamieson's yolk agar even after continuous incubation for periods of up to twelve months, nor has it occurred on media without the addition of killed Myco. phlei. Single colonies were smooth, convex, and glistening; the growth was soft, readily emulsified and of a bright orange colour. Secondary colonies, such as occur frequently with Myco. johnei, were not observed. The colour was present from the time of visible growth, developed in the incubator, and was affected neither by prolonged incubation nor exposure to light at room temperature.

It was apparent that both the Icelandic and the pigmented strains differed from Myco. johnei in at least one of their cultural requirements in that neither would grow in primary culture on a medium containing a low proportion of egg yolk: Dunkin's medium contains 15-20 per cent. of yolk and the modified Finlayson medium, 60 per cent. To test the value of egg yolk in supporting growth, therefore, media were prepared in which the proportion of egg yolk ranged from 15 - 60 per cent.: each medium contained in addition 4 per cent. of glycerin and 1 per cent. of heat-killed Myco. phlei. For the purposes of experiment, it was assumed that the egg white in the medium was inert. These media were inoculated with equal quantities of dilute suspensions of culture in Ringer solution and incubated for three months. The results in the table indicate that growth of either the Icelandic or the pigmented types in primary culture is unlikely on an egg medium containing less than 50 per cent. of yolk, whereas the classical bovine strains are less fastidious and can be grown on media containing only 25 per cent. of yolk.

To compare the pathogenicity of the organisms for laboratory animals with that of Myco. johnei three pigmented and one Icelandic strain were each

TABLE

The growth of varieties of Myco. johnei *on egg media with different proportions of yolk and white*

Inoculum	Growth in media with egg yolk (Y) and egg white (W) in the amounts shown (per cent.)				
	Y = 15 W = 60	Y = 25 W = 50	Y = 37 W = 37	Y = 50 W = 25	Y = 60 W = 15
Myco. johnei (bovine) primary culture	—	+	+	+	+
Icelandic strain, 6th subculture	—	—	+	+	+
Pigmented strain, 6th subculture	—	—	+	+	+
Pigmented strain, 3rd subculture	—	—	—	+	+
Pigmented strain, 2nd subculture	—	—	—	+	+

— = no growth + = growth

each inoculated separately into two guinea-pigs intraperitoneally and one rabbit intravenously. One bovine strain was inoculated similarly as a control. The cultures were grown on modified Finlayson medium for 8 weeks, suspended in Ringer solution and inoculated in a dose of 10.0 mg. The animals were tested intradermally with 0.1 ml. of both avian and mammalian P.P.D. tuberculins (Ministry of Agriculture) at three and again at seven weeks after inoculation, when they were destroyed for examination. In rabbits no response to either tuberculin was produced by any of the strains and in guinea-pigs, although the response was poor, the reaction to avian tuberculin was in each case greater than to mammalian. The result of the autopsy on each animal was similar to that to be expected after the inoculation of a large dose of Myco. johnei or some other Mycobacterium not normally considered pathogenic for laboratory animals. All were in good bodily condition and lesions were not found except in one guinea-pig, which showed one slightly enlarged and caseous mesenteric lymph node. Cultures were prepared from the liver of the rabbits and the spleens and mesenteric lymph nodes of the guinea-pigs. Organisms were grown from the liver of two of the five rabbits, from the lymph nodes of all the guinea-pigs, and from four of the ten guinea-pig spleens, but in each case the number of colonies was small, giving no suggestion of a progressive infection. The pigmented strains showed no alteration in morphology or colouration after passage through the guinea-pig.

Discussion

The isolation in culture of two varieties of Johne's bacilli hitherto considered resistant to cultivation in vitro was accomplished by the use of a medium superior to that formerly employed in work of this nature and provides another example of the value of egg yolk as a major constituent

constituent of media used for the primary isolation of mycobacteria.

It is probable that neither the Icelandic nor the pigmented strains are other than varieties of the classical type of Myco. johnei. The three organisms are the apparent causes of what is, both clinically and pathologically, essentially the same disease in sheep and cattle. The new strains have a uniformly low virulence for laboratory animals. Further, the two varieties share with Myco. johnei its unique requirement of an accessory factor in the form of killed acid-fast organisms in the medium upon which it is grown.

Fundamentally the Icelandic strains appear to differ but little in vitro from Myco. johnei. The difference lies in their inability to grow on a medium which will support the classical type, and their slightly slower rate of growth. On the other hand the ten pigmented strains probably form a definite type or variety of Myco. johnei, and the distinction "ovine type" has already been suggested (Taylor, 1945). Their relationship with Myco. johnei has been described in this paper, but the brilliant colour of the growth and the morphology and consistency of the colonies are all distinct from those of the classical type. It is well known that the Mycobacteria as a genus have a tendency towards pigmentation, but this is not common among the pathogenic species except under certain well-defined conditions. The pigmentation of these cultures, however, appears to be an inherent character as it is present at all stages of growth and is not altered by animal passage. Indeed the orange colour of the lesions of the disease in sheep, which was said by Taylor (1945) to be due to staining with bile, is in all probability caused by the colour of the organisms themselves as they are present in the lesions in very large numbers.

Summary

Johne's disease (paratuberculosis) of sheep may be caused by one of three related organisms: (1) M. johnei, the classical bovine type; (2) a similar organism isolated from sheep in Iceland; and (3) a highly pigmented organism isolated from sheep in Scotland.

Growth of the Icelandic and pigmented strains, hitherto said to be resistant to artificial cultivation, is described for the first time; both are probably varieties of the classical type.

I am indebted to Dr. S. Jamieson, Aberdeen, Dr. P.A. Palsson, Reykjavik, and Mr. J.T. Stamp, Edinburgh, who provided the original material which made this work possible.

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The experimental infection of cattle with varieties of *Mycobacterium johnei* isolated from sheep

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In Press

Taylor (1951) showed that one of three varieties of Mycobacterium johnei might be the cause of Johne's disease of sheep. The first is the classical type as isolated from Johne's disease in cattle, the second a strongly pigmented organism now known to occur in sheep in Scotland, the North of England and in Wales and the third is the cause of Johne's disease of sheep in Iceland. The Icelandic and pigmented strains differ from the classical type in their cultural requirements and neither have as yet been recorded as a cause of disease in cattle.

The work described in this paper was undertaken in an endeavour to confirm these ovine strains as varieties of M. johnei by examining their ability to produce the syndrome of Johne's disease in cattle.

Material and Methods

Experimental Animals

Second generation Red Poll cross Ayrshire calves bred at this Field Station from an attested herd with no history of Johne's disease were used. They were all under the age of four weeks when infected and were thereafter maintained in loose boxes in strict isolation.

Inoculum:

Six pigmented strains, none of which had been subcultivated more than six times since their primary isolation; three Icelandic strains also subcultivated six times with as controls, eight bovine strains recently isolated from apparently normal cattle (Taylor, 1952) were used. Each strain was grown separately for from six to eight weeks on egg-yolk-phlei medium (Taylor, 1950) when each group was mixed to give the three inocula ("pigmented", "Icelandic" and "bovine") and standardised in Ringer solution so that 2 mg. of culture was contained in each ml.

Infection:

Infection:

It was shown previously (Taylor, 1953) that Johne's disease may be reproduced in cattle by the intravenous inoculation of 100 mg. of culture to the young calf; this procedure was adopted in the present experiments. Three calves, Nos. P83, P84 and P87, each received 100 mg. of pigmented culture in a volume of 50 ml., two, Nos. P161 and P177 100 mg. of Icelandic culture, and two Nos. P91 and P97, 100 mg. of bovine culture in a similar volume.

Tuberculin Tests:

The comparative single intradermal tuberculin test using Ministry of Agriculture mammalian and avian P.P.D. tuberculin was applied to the calves every three months after infection.

Examination of Faeces:

Every month after infection the faeces were examined culturally using antiformin and the egg-yolk-phlei medium and microscopically.

Post-mortem Examination:

At autopsy the methods adopted were similar to those described previously (Taylor, 1953). Briefly, the extent of the lesions was noted and the presence of Johne's disease in the alimentary tract was confirmed microscopically and culturally. In addition, using oxalic acid and the egg-yolk-phlei medium cultures were prepared from the liver, spleen and lungs and also from the following lymph nodes:- submaxillary; retropharyngeal; broncho-mediastinal; hepatic; ileo-caecal; and occasionally from the prescapular and the iliac. The bovine strains of M. johnei isolated from these tissues were identified by subcultivation to plain egg, glycerine egg, Dunkin's medium and to the phlei medium. Small acid-alcohol fast organisms which grew only on the last two media were accepted as M. johnei.

The pigmented and Icelandic strains were identified by their ability to grow only on the egg-yolk-phlei medium.

RESULTS

The calves all progressed normally until they were approximately one year old, when the symptoms of the clinical disease, i.e. intermittent diarrhoea, loss of condition and alteration in the coat, began to make their appearance. From fifteen to eighteen months after infection, all the calves, with one exception No.P87, infected with the pigmented type, had developed into typical clinical cases of Johne's disease with emaciation and profuse diarrhoea. The calves infected with the Icelandic and bovine types excreted the organism continuously in their faeces from the first month after infection until the end of the experiment. The two "pigmented" calves which developed clinical symptoms, i.e. Nos. P83 and P85 showed a similar excretion of the organism, but it was only recovered intermittently from No.P87 until the 13th month after infection. These results were obtained by cultural methods, for as has been shown previously (Taylor, 1953), it was impossible to confirm the presence of the disease by the microscopic demonstration of clumps of bacilli in the faeces until clinical signs were becoming evident, i.e. twelve to thirteen months after infection.

The results of the tuberculin tests on these animals is shown in Table I, which again confirms opinions expressed elsewhere (Taylor, 1952) that the comparative tuberculin test may be regarded as insufficiently specific to enable it to be of particular value in the diagnosis of Johne's disease.

All the calves were slaughtered for post-mortem examination from sixteen to eighteen months after infection and all showed extensive lesions of Johne's disease in the alimentary tract. These were most marked in the

TABLE I

TUBERCULIN TESTS

INFECTION	Calf No.	MONTHS AFTER INFECTION					
		3	6	9	12	15	18
PIGMENTED	P83	A 7 M 3	A 5 M 0	A 6 M 1	A 9 M 3	A 5 M 3	A 2 M 0
	P85	A 6 M 3	A 8 M 1	A 9 M 3	A 6 M 3	A 3 M 1	A 2 M 0
	P87	A 7 M 3	A 11 M 1	A 8 M 1	A 7 M 2	A 4 M 2	A M
ICELANDIC	P161	A 7 M 0	A 5 M 1	A 2 M 0	A 3 M 1	A 2 M 0	
	P177	A 8 M 1	A 9 M 2	A 4 M 1	A 2 M 0	A 3 M 1	
BOVINE	P91	A 6 M 3	A 4 M 0	A 4 M 2	A 4 M 1	A 4 M 2	
	P97	A 6 M 3	A 31 M 8	A 15 M 5	A 3 M 1	A 4 M 2	

The figures express the increase in skin thickness in m.m. at the 72nd hour.

A = Avian PPD tuberculin
M = Mammalian "

the animals showing clinical symptoms but even No. P87, which was in good store condition when slaughtered, showed extensive lesions in the small intestine and early lesions in the large intestine. The result of the cultural examination of these animals is shown in Table II and confirms a previous observation (Taylor, 1953) that from clinical cases of Johne's disease the causal organism may be isolated not only from the alimentary tract but from many of the other tissues and lymph nodes of the body.

The disease in sheep caused by the pigmented type of M. johnei is characterised by the orange colour of the lesions in the intestine and this phenomenon was very well shown in Nos. P83 and P85 but to a less extent in P87. In these animals the mucous membrane of the affected parts of the intestine, although greatly thickened and showing the typical corrugations associated with the disease was of a brilliant orange yellow colour. In the other animals the lesions were indistinguishable from those occurring in natural cases of Johne's disease.

The cultural characteristics of the pigmented and Icelandic strains were retained throughout the experiment, i.e. the organisms present in the faeces and in the tissues at autopsy grew only on the egg-yolk-phlei medium and not on Dunkin's medium. The bright orange colour of the colonies of the pigmented type was also unaffected.

SUMMARY

1. The pigmented and Icelandic varieties of M. johnei isolated from sheep have been shown to be capable of producing clinical Johne's disease in cattle.

2. Both varieties retained their characteristic cultural requirements and the orange colour of the pigmented type was unaffected after one passage through cattle.

TABLE II

Recovery of M. johnei in culture from experimentally infected calves

INOCULUM	Calf No.	Small Intestine	Large Intestine	Liver	Lung	Spleen	Retropharyngeal lymph node	Submaxillary lymph node	Broncho-mediastinal	Hepatic lymph node	Ileo-caecal lymph node	Prescapular lymph node	Iliac lymph node
PORCINE	P83	+	+	+	+	-	+	+	+	+	+	-	-
	P85	+	+	+	+	+	+	+	+	+	+	ND	ND
	P87	+	+	-	-	-	-	-	-	-	+	ND	ND
ICELANDIC	P161	+	+	+	+	-	+	-	+	+	+	ND	ND
	P177	+	+	+	+	+	+	+	+	+	+	+	ND
BOVINE	P91	+	+	+	+	+	+	-	+	+	+	-	+
	P97	+	+	+	+	+	+	-	+	+	+	-	+

+ = M. johnei recovered in culture

- = " not " " "

ND = No cultures made

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Experimental Johne's Disease in Cattle

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The calves were castrated when approximately three months old; at no time during the experiment did they show signs of intercurrent disease and worm egg counts were invariably insignificant.

Infection:

A mixture of five recently isolated strains of Mycobacterium johnei was used in an arbitrary dose of 100 mg. to infect each calf. None of the strains had been subcultivated more than four times since their primary isolation and none grew without the addition of heat-killed M. phlei to the medium. They were grown separately for 6 to 8 weeks on Dunkin's medium, when the growth was removed, weighed wet and ground in an agate mortar with Ringer solution so that the final suspension contained a mixture of all five strains in equal parts by weight. The calves were infected as shown in Table 1; those infected per os received the suspension as far back on the tongue as was possible to place it with a pipette; the remainder were inoculated into the jugular vein.

Tuberculin Tests:

The calves were tested every three months after infection. P.P.D. avian and mammalian tuberculin and P.P.D. johnin, all in strengths of 0.4 mg. P.P.D. per ml., and received through the courtesy of Dr. H.H. Green, Weybridge, were used in a single intradermal injection in the side of the neck. The reactions were measured after 72 hours.

Cultural Examination of faeces and tissues:

The methods and medium used in preparing cultures of M. johnei in this laboratory have already been described in detail elsewhere (Taylor, 1950). Briefly, faeces are treated with antiformin and tissue with oxalic acid prior to inoculation on at least five bottles of medium. Initially,

TABLE I

The Experimental Infection of Calves with M. johnei

VOLUME AND ROUTE OF INJECTIONS

Number	Age Infected	Route	Dosage
1 and 2	Birth	Per OS	100 mg. in 1 ml.
3 and 4	Birth	I.V.	100 mg. in 10 ml.
5 and 6	3 Months	Per OS	100 mg. in 1 ml.
7 and 8	3 Months	I.V.	100 mg. in 10 ml.
9 and 10	6 Months	Per OS	100 mg. in 1 ml.
11 and 12	6 Months	I.V.	100 mg. in 10 ml.

Initially, Dunkin's medium was used, but towards the end of the experiment and throughout the post-mortem examinations, an egg yolk medium containing 4 per cent of glycerine and about 1 per cent. of heat-killed M. phlei was used. The bottles were incubated for three months before being discarded as negative, but colonies resembling those of M. johnei were subcultivated to plain egg, glycerine egg and the phlei medium. Cultures of small acid-alcohol fast bacilli which grew only on the last mentioned, after from four to six weeks incubation at 37°C. were accepted as M. johnei.

Cultures were prepared from the faeces of each calf every month after infection. At autopsy a number of tissues were examined as follows:- In the alimentary tract, if lesions were present, cultures were made from the abomasum, duodenum, jejunum, ileum, ileo-caecal valve and large bowel. When lesions were not obvious, the small intestine was divided into approximately 8 ft. lengths from each of which a number of pieces of mucous membrane were removed and mixed prior to grinding with oxalic acid. Thus, from "no lesion" cases fifty to sixty bottles of medium were used for the small intestine alone, apart from the routine cultures from other parts of the bowel. In addition, from each animal cultures were made from the liver, lungs, and spleen, from the submaxillary, retro-pharyngeal, broncho-mediastinal, hepatic and ileo-caecal lymph nodes, and occasionally from the prescapular and iliac lymph nodes.

RESULTS

All the experimental animals were destroyed for examination at intervals between two and three years after infection; none actually died from Johne's disease. For ease of description the experimental results have been grouped under four headings - clinical signs, examination of faeces, tuberculin tests and post-mortem findings.

Clinical Signs:

The clinical syndrome of Johne's disease was eventually shown by four of the twelve animals, Nos.1, 3 and 4 infected at birth, and No.7 infected when three months of age. These animals progressed normally, with the exception of No.3, which was slightly undersized, until about 12 to 15 months after infection, when occasional bouts of diarrhoea, lasting for one or two days, made their appearance. The disease then followed its usual insidious course, with gradual loss of condition and more frequent diarrhoea, until the animals were aged about two years, when they had developed into typical clinical cases of Johne's disease, except that the diarrhoea was never so profuse as the bodily condition might have warranted. Had green food or roots been fed, however, it is possible that the classical type of profuse fluid diarrhoea would have appeared.

The disease in Nos.1, 4 and 7 continued its usual course, but in No.3, at the age of 23 months, suddenly became acute and the animal was destroyed. This bullock had shown well marked loss of condition and intermittent diarrhoea for roughly six months, but on 15th June, 1947, did not feed and on the following day diarrhoea was profuse. On June 17th, the faeces were fluid, black in colour with blood clots and much mucus giving the tail and hind-quarters a black, sticky appearance. The animal was very weak, most unwilling to rise and showed a temperature approaching 103°F. Loss of condition was very rapid; the black diarrhoea with fresh blood and blood clot continued and the animal was destroyed on June 19th. The post-mortem findings are described later.

The remaining eight animals continued to thrive until the end of the experiment when most of them were in good store condition.

Examination of Faeces:

The result of the cultural examination of faeces from each animal at monthly intervals after infection is shown in Table II. The isolation of M. johnei in culture from lightly infected faeces may, however, be so adversely affected by a number of different factors that it is extremely likely that on many occasions the organism was not recovered when it was indeed present in the original material.

Three of the four calves infected at birth began to excrete the organism in their faeces more or less immediately, in contrast to the "three months" group from which no isolation was obtained until eight months after infection. From the "six months" group M. johnei was recovered but once and that from an animal in which, as will be shown subsequently, no organisms were found at autopsy.

Microscopic examination of the faeces was of little value in following the progress of the infection. Films were prepared both directly, by emulsifying a loopful of faeces with normal saline on a slide, and after concentration with antiformin, which has an unfortunate tendency to break up any clumps of organisms that may be present. Single organisms were seen repeatedly throughout the experiment, but not until clinical changes were beginning to become obvious did the diagnostic groups and clumps of bacilli appear in the films.

Tuberculin Tests:

The result of tuberculin and johnin tests performed every three months after infection is shown in Table III. These figures are not comparable with those obtained in the field nowadays in this country, as it should be borne in mind that they were begun in 1945, before the

TABLE II

Cultural Examination of Faeces of Artificially Infected Cattle for M. johnei

	Age in Months																														
Nos.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	J	+	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D							
2	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+	-	+	D				
3	J	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	D								
4	J	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D				
5			J	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	+	-	+	-	D					
6			J	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	+	-	D					
7			J	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D								
8			J	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	
9						J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D
10						J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D		
11						J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D				
12						J	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D

\+ = M. johnei recovered in culture

J = Injected

D = Destroyed

TABLE III

Tuberculin and johnin tests on cattle experimentally infected with M. johnei

The figures express the increase in skin thickness at the 72nd hour after injection

A = avian P.P.D. tuberculin

M = mammalian P.P.D. tuberculin

J = P.P.D. johnin

I = infected

Number		Age in Months										
		0	3	6	9	12	15	18	21	24	27	30
1	A	I	1	6	5	4	9	9	3	6		
	M		0	1	1	1	0	1	1	0		
	J		2	0	2	2	4	2	1	0		
2	A	I	1	10	12	**10**	14	7	4	7		
	M		0	0	2	**1**	1	1	1	2		
	J		0	4	7	7	**11**	**5**	**3**	**4**		
3	A	I	1	5	9	6	1	6	3			
	M		0	1	1	1	1	1	1			
	J		0	0	4	1	1	1	2			
4	A	I	7	13	9	8	8	6	3	2	4	5
	M		2	2	1	0	2	0	0	2	1	0
	J		2	1	3	2	2	0	1	1	1	1
5	A		I	6	3	1	17	14	1	6		
	M			1	1	0	2	3	0	0		
	J			1	0	1	13	7	4	4		
6	A		I	3	2	2	1	2	6	8	10	
	M			1	0	0	1	0	2	0	1	
	J			3	0	1	0	2	4	4	6	
7	A		I	2	14	11	6	6	4			
	M			0	2	1	0	0	1			
	J			1	8	4	3	3	4			
8	A		I	3	9	7	6	3	0	1	3	3
	M			0	1	0	3	2	0	1	0	0
	J			1	3	1	2	3	0	1	1	0
9	A			I	1	1	2	1	2	2	1	4
	M				0	0	0	0	0	1	0	0
	J				1	1	3	0	1	1	1	3
10	A			I	1	1	2	1	2	8	4	
	M				1	0	1	1	1	1	2	
	J				1	0	1	1	4	8	4	
11	A			I	12	2	3	3	3	1	1	
	M				0	1	0	2	0	1	0	
	J				4	4	2	1	0	1	1	
12	A			I	4	6	5	1	0	1	0	1
	M				0	0	0	1	1	0	1	0
	J				1	1	2	2	0	0	1	3

the introduction of the "single" intradermal test as the official test, and that "equal strength" P.P.D. tuberculins were used, i.e. all contained 0.4 mg. P.P.D. per ml., whereas the official tuberculin now contains 0.5 mg. of avian P.P.D. and 2.0 mg. per ml. of mammalian P.P.D. respectively. Consequently, the reactions shown in the table particularly those to mammalian are smaller than might be expected with the present strength of tuberculin. The table does show, however, a superiority of P.P.D. avian tuberculin to P.P.D. johnin in eliciting a reaction in infected animals and also the fact that the allergic response is usually at its height during the earlier stages of the infection and tends to fade out with the onset of clinical signs of the disease.

Post-mortem Findings:

The result of the post-mortem examinations is shown in Table IV, which indicates that of the twelve calves, clinical disease developed in four, Nos. 1, 3, and 4 infected at birth and No.7 infected when three months old. The remaining animals were in good condition when slaughtered but typical lesions of the disease were present in No.2, infected at birth, and Nos. 5 and 6 infected when 3 months old. Very small numbers of M. johnei were isolated in culture from two animals Nos. 8 and 10 in which lesions of the disease were not found either macroscopically or histologically, whereas in the remainder, Nos. 9, 11 and 12, injected when 6 months old, infection was not demonstrated.

Calf No.3 developed the disease in an acute form after having shown clinical symptoms for some months and was destroyed. At autopsy there was an almost complete absence of fat and lesions were confined to the alimentary tract. The abomasum showed a few petechial haemorrhages and gross lesions of Johne's disease were present from the pylorus to the anus. The small

TABLE IV - POST-MORTEM EXAMINATION OF CATTLE ARTIFICIALLY INFECTED WITH M. JOHNEI

Number	Age Infected in months	Clinical Signs	Macroscopic lesions	Age in Months	RECOVERY OF M. JOHNEI IN CULTURE												
					Abomasum	Small Intestine	Large Intestine	Liver	Spleen	Lung	Mesenteric lymph node	Hepatic lymph node	Bronchomediastinal lymph node	Submaxillary lymph node	Retropharyngeal lymph node	Iliac l.node	Prescapular lymph node
1	0	YES	YES	25	-	+	+	+	-	+	+	+	+	+	+	ND	ND
2	0	NO	? YES	26	ND	+	-	-	-	-	+	-	-	-	-	ND	ND
3	0	YES	YES	22	+	+	+	+	+	+	+	+	+	+	+	ND	+
4	0	YES	YES	34	+	+	+	+	-	+	+	+	+	+	+	ND	+
5	3	NO	YES	26	-	+	-	-	-	-	+	-	-	-	-	ND	ND
6	3	NO	YES	29	-	+	-	+	-	-	+	+	+	-	+	ND	ND
7	3	YES	YES	24	-	+	+	+	-	+	+	+	+	-	+	-	ND
8	3	NO	NO	34	-	1 +	-	-	-	-	2 +	-	-	-	-	ND	ND
9	6	NO	NO	32	ND	-	-	-	-	-	-	-	-	-	-	ND	ND
10	6	NO	NO	30	ND	-	-	-	-	-	1 +	-	-	-	-	ND	ND
11	6	NO	NO	28	ND	-	-	-	-	-	-	-	-	-	-	ND	ND
12	6	NO	NO	31	ND	-	-	-	-	-	-	-	-	-	-	ND	ND

+ = M. Johnei recovered in culture
- = M. Johnei not recovered
1 = 1 colony; 2 = 2 colonies
ND = Not done

small intestine was almost empty but the last 15 to 20 feet were abnormally thickened and showed bright red blood in the folds of mucous membrane, the contents being an adherent mixture of blood and mucus. The terminal portion of the ileum showed definite ulceration, which was confirmed histologically, a number of eroded areas, up to 2 cm. in diameter being present. The appearance of the large intestine was like that of the ileum; there was fresh blood in the folds of intestine and the contents were similar. A shallow irregularly edged ulcer, approximately 3 cm. across, was present in the caecum near the valve. The mesenteric lymph nodes were, in many cases, swollen and oedematous. It is shown in Table IV, that M. johnei was recovered from all the tissues from which cultures were made; in fact an almost confluent growth was obtained from tissues such as the liver and lungs, so that a condition resembling bacteraemia may have been present in this animal.

When it became apparent from these experimentally infected cattle showing clinical symptoms of Johne's disease, that M. johnei might be isolated in culture from tissues other than those of the alimentary tract, a number of naturally occurring cases of the disease were examined with the result shown in Table V. This indicates that, from clinical cases of Johne's disease in cattle M. johnei may be regularly isolated not only from the intestine and its associated lymph nodes, but also from many of the other tissues of the body.

DISCUSSION

It has been recognised for many years that young cattle may be more susceptible to infection with M. johnei than older animals. This is well borne out by the experiments described in this paper, which have also established that a single inoculum of 100 mg. of culture of recently isolated

TABLE V

Recovery of M. johnei in culture from naturally occurring clinical cases of Johne's Disease

Number	Small Intestine	Large Intestine	Liver	Spleen	Lung	Mesenteric lymph node	Hepatic lymph node	Broncho-mediastinal lymph node	Submaxillary lymph node	Retropharyngeal lymph node	Iliac lymph node	Preauricular lymph node
A.88	+	+	+	-	-	+	+	+	+	+	ND	ND
A.90	+	+	+	+	-	+ T.B.	+	+	- + T.B.	+	ND	ND
W.I	+	+	+	+	+	+	+	+	+	+	ND	ND
662	+	+	+	+	+	+	+	+	+	+	ND	ND
J.52	+	+	+	+	+	+	+	+	-	+	ND	ND
C.2	+	ND	+	+	+	+	+	+	+	+	ND	+
J.140	+	+	+	ND	+	+	+	+	+	+	ND	ND
F.S.S.	+	ND	ND	ND	ND	+	+	+	+	+	+	+
49505	+	+	+	-	-	+	+	-	+	+	ND	ND
1418	+	+	+	-	+	+	+	+	+	+	ND	+
452	+	+	+	-	-	+	+	+	-	-	ND	+

+ = M. johnei isolated. — = negative. N.D. = no cultures made.

isolated strains will so infect the young calf that extensive lesions of the disease, if not always the complete clinical syndrome, may be reproduced. Older animals, i.e. those of 6 months of age, as was to be anticipated, were not so infected. It is conceivable that the young calf might be infected by a very much smaller inoculum than 100 mg. and that adult animals might also be infected by larger or repeated doses, but the primary purpose of the experiment, namely the determination of a suitable infecting dose, was achieved. There is no known method of assessing the virulence of individual strains of M. johnei; therefore, it would appear essential, in infection experiments, to use recently isolated strains of the organism. To establish with certainty that a bovine animal is free from infection with M. johnei is not practicable. The sheer bulk of the bovine intestine and its associated lymph nodes is so great that only a minute proportion may be examined culturally or microscopically and further, the difficulties inherent in the primary isolation of M. johnei militate against its demonstration when present in minimal numbers.

The number of animals used in this experiment was of necessity, small, but was sufficient to establish several features not previously recognised, or at least only suspected, regarding Johne's disease. Thus it has been confirmed that an infected animal, in apparent good health, may excrete the organism in the faeces for many months before either clinical signs become apparent, or infection can be demonstrated by microscopic examination of the faeces. This is of obvious importance in the control of the disease and it is unfortunate that the technical difficulties of the method, i.e. by culture from the faeces, are at present so great as to render it of little value as a routine method of diagnosis.

The tuberculin tests of these animals cannot properly be compared with present-day results, but again they tend to confirm an impression expressed elsewhere (Taylor, 1952) that allergic tests are of little value in the diagnosis of Johne's disease.

It has long been understood that in the animal body M. johnei was confined to the lesions of the disease in the intestine and to its associated lymph nodes. This opinion has rarely been disputed, except by Alexjeff-Goloff (1929) who "startled current opinion" (Levi 1948) by recording the isolation of M. johnei from different organs and the milk, blood and urine of advanced cases of the disease. Other recorded instances of the isolation of the organism from extra-intestinal sites have been very few in number and were reviewed by Levi (1948) who was, however, able to obtain M. johnei in culture twice from the pharyngeal lymph nodes and once each from the tonsil and spleen of naturally affected cows. The present work, however, has established that M. johnei may be regularly recovered in culture from almost any of the lymph nodes or abdominal or thoracic viscera of cattle showing clinical signs of the disease. Two distinct factors have probably been responsible for this result. First, in clinical cases, the organism is commonly present in the mesenteric lymph nodes in large numbers; it is known to be resistant to destruction within the animal body and may through sheer weight of numbers occasionally escape from the lymph nodes into the lymph stream, from which a mild bacteraemia may be set up. In the second place, the use of oxalic acid and an egg-yolk-phlei medium (Taylor, 1950) for the primary cultivation of M. johnei is greatly superior to methods previously used; in other words the technical methods employed by many of the earlier workers were insufficiently delicate to demonstrate organisms unless these

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these were present in quantity, as in the intestine and mesenteric lymph nodes.

SUMMARY

1. The lesions and clinical syndrome of Johne's disease may be reproduced in cattle by the intravenous inoculation or *per os* administration of 100 mg. of recently isolated culture of *M. johnei* to young calves.

2. Calves so infected excrete *M. johnei* in their faeces for many months before clinical signs of the disease become apparent or the organisms can be demonstrated microscopically.

3. *M. johnei* may be recovered in culture, not only from the intestine and its associated lymph nodes but also from many of the other tissues of clinical cases of Johne's disease in cattle.

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Observations on the Incidence of Infection with *Mycobacterium johnei* in cattle.

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The incidence of infection with M. johnei (Johne's bacillus) among cattle in this country has not been accurately determined. It is the purpose of this paper to record some observations on the infection rate with this organism in 243 apparently normal animals consigned to the Ministry of Food for human consumption.

Taylor (1945) has shown that extensive lesions of Johne's disease may be present in the intestine of apparently healthy sheep; more recently (unpublished) that the same phenomenon may be observed in experimentally infected cattle and further, that M. johnei may be isolated in culture from bovine intestine and ileo-caecal lymph nodes which show no macroscopic lesions of the disease. It seemed probable, therefore, that further information might be gained by the cultural examination of a large number of nodes from apparently normal animals that would be a representative sample of the adult bovine population. It has been our experience that in lightly infected cattle, or from animals in the so-called incubation period, organisms may be isolated with greater regularity from the ileo-caecal lymph node than from the intestine. This finding does not of course denote that infection is more regularly confined to the lymph node but merely that the sheer bulk of the bovine intestine renders impossible the cultural examination of its entire mucous membrane. Further, the lymph node is readily studied both culturally and histologically and the routine of the slaughterhouse is not upset by its collection. Accordingly, arrangements were made to obtain from an abattoir in Berkshire, twice weekly, ileo-caecal lymph nodes from graded cattle and to examine them culturally for M. johnei. Lymph nodes were not taken from animals showing any symptoms suggestive of Johne's disease.

METHODS

METHODS

A modification of the method originally described by Corper and Uyei (1930) for the isolation of tubercle bacilli was used. The lymph nodes were freed from fat and halved. One half was fixed for histological examination; the other was dipped in alcohol and flamed twice, then ground in a mortar with sand and a 5 per cent. solution of oxalic acid in distilled water. The resultant suspension was filtered through four layers of butter muslin, incubated for 30 minutes in a water bath at 37°C. and centrifuged for 20 to 30 minutes at approximately 3,000 R.P.M. The unwashed sediment was then thickly sown on each of five screw-capped bottles of an egg medium containing approximately 1 per cent. of heat killed M. phlei. The bottles were incubated at 37°C. for 12 weeks before being discarded.

Colonies resembling those of Johne's bacillus were subcultivated to plain egg, glycerine egg and the phlei medium. Cultures of small, acid-alcohol fast bacilli, which grew only on the last mentioned, after four to six weeks' incubation at 37°C., were accepted as M. johnei.

RESULTS

During the four months ending on December, 31st, 1948, cultures were prepared from the ileo-caecal lymph nodes of 243 different cattle. M. johnei was recovered from 37, or 15 per cent., a figure which suggests that the limits for larger samples will fall between 10 and 20 per cent. (Mainland, 1948). One hundred and fifty-four nodes were obtained from cows, defined as animals which were, or had been, in milk, and of these, 22, or 14 per cent., were positive. The remaining 89 lymph nodes were derived from heifers, bullocks and bulls and from these M. johnei was recovered from 15, or 17 per cent. No calves were examined.

An acid-alcohol fast organism as yet unidentified was, in addition,

addition, recovered from one lymph node.

DISCUSSION

The preliminary results of the survey described here have shown an unexpectedly high incidence of infection with M. johnei in apparently normal cattle. The true significance of this is not readily assessed in the present state of our knowledge of Johne's disease. If it be assumed that all field strains of M. johnei are of more or less equal virulence for cattle, then, as 15 per cent. of 243 animals were found to harbour the organism without showing the symptoms usually associated with the disease, the opinion may be expressed that M. johnei, while more highly infective than previously thought, is possibly not particularly pathogenic for cattle. There are no available figures regarding the incidence of clinical Johne's disease, but it is unlikely, if, of a random sample of the adult bovine population, such as has been examined here, 15 per cent. become clinical cases. It may be argued that many cattle do not live long enough to develop clinical signs, but another explanation may be, that of a number of animals exposed to M. johnei at an early age, many will eventually rid themselves of infection, or may harbour the organism as a commensal over a long period without showing symptoms of the disease.

It is well known that the clinical syndrome of Johne's disease is not readily produced artificially in cattle but it has been shown experimentally (unpublished data) that the majority of calves given a large dose of M. johnei become infected, but by no means do all of these develop symptoms. In other words, M. johnei may belong to that varied group of parasites which exist as normal inhabitants of the body, c.f. Staph. aureus, Strep. pneumoniae,

Strep. pneumoniae, Ery. rhusiopathiae, Actinomyces bovis, and become pathogenic, only under certain environmental conditions.

The infection of cattle with M. johnei is one of the known causes of non-specific sensitivity to mammalian tuberculin. The unexpectedly high infection rate observed in this survey of apparently normal animals suggests that this organism may be a more important cause of such sensitivity than has hitherto been believed.

The investigation is being continued.

SUMMARY

M. johnei was isolated in culture from the ileo-caecal lymph node of 37 out of 243 graded cattle consigned to a slaughterhouse.

Acknowledgments

Thanks are due to the management and staff of the Ministry of Food Slaughterhouse, Newbury, without whose co-operation the investigation would be impossible, and to the Director of the Field Station, Dr. W.S. Gordon, for the interest he has taken in this work.

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Further Observations on the Incidence of Infection with *Mycobacterium johnei* in cattle

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In a preliminary report of a survey of the incidence of infection with M. johnei (Johne's bacillus) in apparently normal cattle, Taylor (1949) recovered the organism in culture from the ileo-caecal lymph node of 37 of 243 graded animals, taken at random from those slaughtered for human consumption by the Ministry of Food at an abattoir in Berkshire.

This paper records the amplification and completion of this survey.

MATERIAL AND METHODS

The reasons for selecting the ileo-caecal lymph node for examination in a survey of this nature were described previously; the method of cultivation and the medium used, i.e. preliminary treatment of the tissue with oxalic acid followed by incubation on an egg yolk medium containing killed M. phlei, have been published in detail elsewhere (Taylor, 1950).

The lymph nodes were derived from cattle from two different sources; one, the "Slaughterhouse Group", was a random sample of graded animals slaughtered for human consumption by the Ministry of Food in Newbury. None of these animals showed symptoms suggestive of Johne's disease and if, in the laboratory, macroscopic lesions resembling those of tuberculosis were found in the tissue, that lymph node was not examined further. The remainder, the "Estate Group," whose tuberculin test history was available for examination, were attested cattle discarded mainly because of sterility or as "poor milkers" from an estate in Berkshire. On this estate Johne's disease has not been a herd problem for many years, only two clinical cases having occurred during the last four years, one in 1948 and another in 1950.

The identity of M. johnei is established in this laboratory by subcultivation of the primary culture to plain egg and glycerine egg media and to the phlei medium. Strains of small acid-alcohol-fast bacilli which

which grow only on the last mentioned after four to six weeks' incubation at 37°C. are accepted as M. johnei. This method of identification, i.e., solely by cultural means, may be open to objection, but is the only practicable method at present available when large numbers of strains are handled. However, certain of the strains isolated in this investigation, selected at random, were used subsequently to infect cattle and in these animals they produced the classical syndrome of Johne's disease.

RESULTS

Slaughterhouse Group: During the 11 months from September, 1948, to July, 1949, the ileo-caecal lymph nodes of 665 cattle were examined culturally. M. johnei was isolated from 101, or 15 per cent.; 381 of these animals were cows defined as animals which were, or had been, in milk, and from these M. johnei was recovered 55 times or from 14 per cent. The remaining 284 were bulls, bullocks or heifers, and of these 46, or 16 per cent. were positive. There is obviously no significant difference in the degree of infection of these two groups.

M. tuberculosis (bovine type) was isolated from three of the lymph nodes despite the facts that any lymph nodes showing lesions of tuberculosis were discarded and that the medium containing 4 per cent. of glycerine was unsuitable for the primary cultivation of bovine tubercle bacilli. These strains were identified by the inoculation of guinea-pigs, rabbits and hens. From two other lymph nodes, M. tuberculosis (avian type) was isolated; each of these strains was shown to be virulent, in that both were capable of producing lesions of tuberculosis and death in inoculated hens.

Estate Group: Cultures were prepared from the ileo-caecal lymph

lymph nodes of 136 animals, the great majority of which were barren cows, between December, 1948, and February, 1952. M. johnei was recovered from eight, or 6 per cent. The tuberculin test history of these infected animals is shown in the table and it is clear that this record would have been of but little value in their selection.

An unidentified strain of acid-alcohol-fast bacillus was isolated from another animal (No. K80) born in August, 1946, and slaughtered in July, 1949. During its lifetime it was tested three times with avian and mammalian tuberculin and on no occasion showed an increase in skin thickness greater than 1 mm. The organism developed a typical mycobacterium-like colony on the usual egg media after seven days' incubation at 37°C. and morphologically was a long slender bacillus resistant, for at least two minutes, to decolorisation by 2 per cent. hydrochloric acid in absolute alcohol. It grew readily on coagulated egg white, a medium said by Finlayson (1946) to be almost incapable of supporting growth of the pathogenic mycobacteria. Biologically the organism was non-pathogenic, in that no macroscopic lesions developed in guinea-pigs inoculated subcutaneously or intraperitoneally with 5 mg. or 10 mg. of culture; these animals, however, reacted to the intradermal application of both avian and mammalian tuberculins up to 20 weeks after inoculation. The strain has been recovered in culture from the guinea-pig spleen, 63 but not 147 days after inoculation. Hens given 10 mg. of culture intravenously showed no ill effects, no macroscopic lesions were demonstrable at autopsy and the tuberculin reaction could only be described as "doubtful", yet the organism could be recovered in culture from the spleen up to nine months after inoculation.

TEST HISTORY OF NORMAL ANIMALS FROM WHICH *M. johnei* WAS ISOLATED

Number, date of birth	Tuberculin tests							Date slaughtered
	1944	1945	1946	1947	1948	1950	1951	
K.91 19.9.46	—	—	—	A 7 – 7 M 8 – 7	A 10 – 15 M 9 – 14 A 10 – 17 M 9 – 16	—	—	4.7.49
J.90 13.7.45	—	A 4 – 5 M 4 – 4 A 5 – 6 M 5 – 5	A 9 – 15 M 10 – 14	A 7 – 8 M 6 – 7	A 8 – 11 M 8 – 9 A 8 – 8 M 8 – 8	A 6 – 7 M 6 – 7	—	12.2.51
J.91 14.7.45	—	—	A 5 – 6 M 5 – 6 A 8 – 8 M 7 – 9 A 7 – 7 M 6 – 6	A 8 – 9 M 7 – 9	A 8 – 9 M 9 – 10 A 9 – 9 M 8 – 9 A 7 – 7 M 8 – 8	A 7 – 13 M 6 – 11	A 7 – 10 M 7 – 8	15.5.51
P.60 16.4.50	—	—	—	—	—	A 7 – 8 M 7 – 8	—	2.7.51
37 5.9.36	A 6 – 8 M 6 – 7	A 5 – 9 M 5 – 6 A 7 – 12 M 6 – 11 A 5 – 7 M 5 – 7	A 5 – 6 M 5 – 7 A 6 – 9 M 6 – 8	A 6 – 6 M 5 – 6	A 6 – 8 M 7 – 7 A 6 – 7 M 6 – 7	A 6 – 7 M 6 – 8	A 6 – 7 M 6 – 6	9.7.51
64 8.5.37	A 6 – 7 M 7 – 7	A 5 – 7 M 5 – 6 A 6 – 7 M 6 – 7 A 5 – 7 M 5 – 6	A 6 – 10 M 6 – 10	A 5 – 11 M 5 – 9	A 5 – 6 M 5 – 5 A 7 – 8 M 6 – 6	A 6 – 7 M 8 – 9	A 6 – 8 M 6 – 6	26.11.51
L.16 7.3.47	—	—	—	A 4 – 5 M 3 – 3	A 9 – 9 M 9 – 9 A 9 – 9 M 8 – 9	A 8 – 8 M 7 – 8	A 6 – 9 M 6 – 7	26.11.51
M.178 2.12.48	—	—	—	—	A 6 – 6 M 6 – 6	A 8 – 9 M 7 – 7	A 8 – 9 M 8 – 8	11.2.52

A = Avian PPD tuberculin. M = Mammalian PPD tuberculin.

DISCUSSION

Two fundamental points in connection with the work of this survey will bear further emphasis. Firstly, as far as could be judged, the cattle examined were free from clinical evidence of Johne's disease; secondly, since the animals were apparently normal it is probable that M. johnei was present in the tissue in very small numbers, and since the organism is also not readily cultivated in the laboratory, the positive results obtained, i.e. 15 per cent. for the slaughterhouse group and 6 per cent. for the estate group, probably do not represent the full infection rate. It was suggested previously (Taylor, 1949) that M. johnei might be regarded almost as a commensal, in that it is only potentially pathogenic for cattle, a view that is supported by the further results described in this paper.

The eight animals of the estate group from which M. johnei was isolated are of interest in that their tuberculin test history is known. They are very few in number, but the table supports the view that the comparative tuberculin test, when applied annually as in attested herds, is almost valueless in the diagnosis of infection with M. johnei. It has already been found by Dr. S.J. Edwards (personal communication) that cattle may spend their lives in attested herds without reacting to either tuberculin or to johnin and yet succumb to clinical Johne's disease. Matthews (1951) was also of the opinion that avian tuberculin was too erratic to be used as a reliable guide to infection, and Green (1946) has stated "If the comparative test shows an infection to be non-mammalian other means than tuberculin testing have to be adopted to show whether that infection corresponds to Johne's disease." It would appear that precise evidence of the usefulness or otherwise of the tuberculin test in the diagnosis

diagnosis of infection with M. johnei will be forthcoming only when large numbers of cattle can be tested at frequent intervals over a period of years and can finally, at slaughter, be carefully examined.

SUMMARY

M. johnei was isolated in culture from the ileo-caecal lymph node of 101, or 15 per cent., of 665 graded cattle consigned to a slaughterhouse, and from eight, or 6 per cent., of 136 adult cattle surplus to the requirements of an estate on which clinical Johne's disease as such does not exist.

Acknowledgments

Acknowledgments are due to the management and staff of the Ministry of Food Slaughterhouse, Newbury, whose co-operation at all times has been of the greatest value.

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Johne's Disease - Its Diagnosis and Control

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The Annual Congress of the National Veterinary Association last discussed the subject of Johne's disease in 1933, when a paper was presented by Dr. Minett. He reviewed much of the knowledge of the disease at that time and laid special emphasis upon differential diagnosis and the use of johnin. While the years since 1933 have seen the development and widespread use in veterinary science of the antibiotics PPD tuberculins and of S.19 vaccine and while vast strides have been made in the control of streptococcal mastitis, tuberculosis and contagious abortion by the use of these products there has been no comparable advance in our knowledge of the control of Johne's disease.

By the term "Johne's Disease" is currently understood a chronic disease of cattle characterised by progressive emaciation and diarrhoea, but it is now recognised that the actual disease is considerably less common in cattle than is infection by Mycobacterium johnei per se, as it is now known that a high proportion of animals, in what appears to be normal health, may in fact be infected by this organism (Taylor, 1949).

This paper has been written as an account of the trend of thought on Johne's disease as expressed in the literature since 1933. It shows that despite some recent advances in our knowledge of the incidence of the infection and of the bacteriology of the disease in cattle and sheep, the deficiencies in our knowledge of its diagnosis and consequently its control are almost as great as ever.

Diagnosis During Life

Faeces Examination:

The diagnosis of Johne's disease by the clinical signs and microscopical examination of faeces is well known. It has been our experience, however, that one is unlikely to find the groups of organisms which alone will confirm

confirm the diagnosis in stained films of faeces in the absence of clinical signs of the disease. Working with experimental cattle, we did not find groups of bacilli in the faeces until the animals were obviously not in normal health. It is our practice, with Levi (1948a) and Doyle and Spears (1951), to examine direct smears of faeces stained by Ziehl-Neelsen, as other methods tend not only to concentrate the Johne bacilli but also the acid-fast saprophytes which may be present in very large numbers and distract the eye from the much smaller Johne bacilli; further, the diagnostic groups tend to get broken up after concentration with antiformin or ether.

Cultural examination of faeces, however, using antiformin and a reliable medium (Taylor, 1950), will reveal the presence of organisms many months before these become apparent in stained films. The method has been used with success at Compton in tracing the infection in experimental cattle and in natural cases where organisms could not be found microscopically. It cannot be too strongly emphasised, however, that this method is of little value as a routine diagnostic procedure. It consumes a great deal of time in the laboratory and even when successful is slow; a positive result can rarely be given before four to six weeks and a negative answer is of little or no value.

Blood Examination:

It was shown by Stewart, McCallum and Taylor (1945) that in Johne's disease in both cattle and sheep the blood magnesium is low and may be very low and it was suggested that this test might be of value in establishing a diagnosis. However, since a low blood magnesium may have a variety of causes, more work on this aspect of the problem is required to determine the practical value of this test in the diagnosis of the disease.

Serological Tests:

It is well known that infections by the pathogenic mycobacteria cannot be differentiated by serological methods. In certain circumstances, however, the complement fixation test may be of considerable value, as Sigurdsson, Vigfusson and Theodors (1945) have shown in Iceland. There, Johne's disease in sheep is a recently introduced infection and has spread rapidly, but by means of a complement fixation test using an antigen derived from infected intestinal mucosa, infected animals may be detected with some considerable degree of accuracy. Positive reactions to Sigurdsson's test are obtained, however, with sera from cases of tuberculosis and leprosy in man and it is therefore, in common with other similar tests, not specific for Johne's disease. In testing cattle in Great Britain, where the incidence of both tuberculosis and of "non-specific" infections is known to be high, there may be considerable difficulty in the interpretation of such a test.

Allergic Tests:

Ever since Olaf Bang (1909) announced that cattle with Johne's disease reacted to injections of avian tuberculin and Twort and Ingram (1913) first announced the production and use of johnin, the advantages of one have been argued against the other. It is not proposed to discuss the matter at length here, but it should be realised that there are many difficulties in the production of a specific product. Firstly, to prepare johnin in a manner similar to tuberculin, it is essential to use a strain of M. johnei that has been adapted for growth on the surface of a synthetic medium, free from M. phlei or its extracts or tuberculin. In other words, an old laboratory strain is used. Now the metabolism of such an "adapted" strain is very different from that of the organisms as first isolated from cattle; whether such strains will still infect cattle is not known, and whether the

the johnin produced from them is the same as the hypothetical product of a recently isolated strain is also unknown. In the standardisation of johnin the method originated by Glover (1941), i.e., the use of guinea-pigs sensitised by the inoculation of M. johnei suspended in liquid paraffin, is commonly used. It is economical and the reactions obtained can be measured with some degree of precision, but Green (1946) believes that the specificity of johnin may be greater in the artificially sensitised guinea-pig than in cattle. He has shown, using PPD material, that in guinea-pigs only 3 units of avian PPD are required to match 1 unit of johnin and vice versa and stated, "Limited tests on cattle suggest that the difference is even smaller with that species and much the same reactions are produced by both PPDs in non-specific infections. Hence a johnin test adds nothing to the information gained in the ordinary comparative test using mammalian and avian PPDs. If the comparative test shows an infection to be non-mammalian, other means than tuberculin testing have to be adopted to show whether that infection corresponds to Johne's disease, ..."

On the other hand, there are numerous references in the literature to the testing of cattle in infected herds with johnin, and many authors have found that the percentage of positives to their johnin test was as high as 30 or even more, although the clinical incidence of the disease might be low. Similarly it is well known that many herds show an equally high percentage of "non-specific" reactors to the comparative tuberculin test. It is somewhat outside the scope of this paper to discuss non-specific sensitivity to the tuberculin test, but it has been shown recently that from 15 per cent. of what are to all intents and purposes healthy cattle, i.e. graded cattle slaughtered for the Ministry

Ministry of Food in Newbury, M. johnei may be isolated in culture from the ileo-caecal lymph node (Taylor, 1949; 1951, in preparation). As it is possible that any cultural test for the detection of M. johnei is little more than 50 per cent. accurate when positive, it is conceivable that the true figure of infection is in the region of 25 per cent. to 30 per cent., a figure which corresponds very closely to that given as the number of positive reactions to johnin in infected herds. Hence it may be possible that the sensitivity produced to either johnin or avian tuberculin, in fact, may be evoked by a true infection with M. johnei in the majority of so-called "non-specific" reactors. It is unfortunate that most work on the testing of cattle with johnin has been done on commercial cattle and not on experimentally infected animals with a known history. Our experience with the latter at Compton is as yet very limited, but there is a tendency in these animals for the reactions to avian tuberculin to be well marked and at their height six to 12 months after infection, but they tend to fall with the onset of clinical signs. Mammalian tuberculin also causes a reaction; it is never so severe as that to avian, but taken by itself might often be regarded as positive. On the other hand, we have isolated M. johnei in culture from the lymph nodes of apparently normal animals whose test history was known, and which at no time during their lives have reacted to either tuberculin.

Johne's Disease in Sheep

Johne's disease in sheep in this country perhaps may be a more frequent cause of loss than is generally realised, but it commonly occurs sporadically in a flock and its presence may be unsuspected. McEwen (1939), however, has described an outbreak in which serious losses occurred. He found the johnin and avian tuberculin tests unreliable and although organisms may be

be found in the faeces of affected animals, the diagnosis may be more readily established by the slaughter and post-mortem examination of one of the affected animals.

Post-mortem Diagnosis

The confirmation of a diagnosis of clinical Johne's disease may normally be obtained at autopsy, although the greatly thickened "hose pipe" type of intestine is not now quite so commonly seen, probably because in an endeavour upon the part of the owner to salvage the carcase, many animals are disposed of before the lesions become so advanced.

The difficulty arises when no characteristic lesions are found. A slightly flushed surface of the mucous membrane of an otherwise normal intestine, with much viscid mucus and petechial haemorrhages, are said to be indicative of Johne's disease, but in our experience these are often unreliable signs of infection. Petechial haemorrhages, on the crest of the folds of the mucous membrane may indicate the site of early lesions but they may also be caused by the method of slaughter as they are present almost invariably in the intestine of cattle that have been stunned and pithed before bleeding. Early lesions may be difficult to distinguish macroscopically from normal bowel and recourse must be made to microscopical or cultural methods. Microscopic examination of stained smears of the mucous membrane will confirm the diagnosis but if lesions are not obvious their preparation and thorough examination is a laborious process and, further, very few organisms may be present. Working at Compton with experimental cattle we have found the cultural method to be more satisfactory in such cases. If no lesions are found the intestine is divided into convenient lengths of from 6 to 10 feet. From each of these, ten to 15 small pieces of mucous membrane are removed with scissors, mixed and ground with oxalic

oxalic acid to control contamination and sown on several bottles of a suitable medium (Taylor, 1950). Thus 50 to 60 bottles of medium may be used for the small intestine alone. The process is both laborious and expensive in time and material, but has yielded good results in experimental animals. Alternatively, cultures may be made from the ileo-caecal lymph node as we have found that in every case we have examined so far, when M. johnei was recovered in culture from the small intestine, it was also present in the lymph node, even when the number of organisms was very small indeed. Thus from one experimental animal, a single colony of M. johnei was recovered from over 60 bottles of medium inoculated from the small intestine, but it also grew in culture from the lymph node. A series of films or impressions from the ileo-caecal lymph node, therefore, may be of some value in the post-mortem examination of field cases and is considerably less laborious than the microscopic examination of the intestine.

The early workers on Johne's disease believed that in the animal body the causal organisms were confined to the lesions of the disease in the intestine and to its associated lymph nodes and this opinion has never been questioned seriously. Various authors, whose work in this connection was reviewed by Levi (1948b), have described its occasional isolation from other sites but none has described the infection as being widespread in the body. It has become the custom at Compton when examining cattle at autopsy to prepare cultures not only from the intestine and the mesenteric lymph nodes but also from the liver, lung and spleen, and from the submaxillary, retropharyngeal, hepatic and broncho-mediastinal group of lymph nodes and occasionally from the iliac, prescapular and supramammary. By the use of oxalic acid to control contamination and a suitable medium, M. johnei was

was isolated with almost unfailing regularity from all these sites. In view of the fact that these isolations had been made from cattle infected experimentally, a number (actually 15) of field cases have been similarly examined with exactly the same result. In other words, M. johnei may be recovered in culture from clinical cases of the disease not only from the classical sites but also from many of the other tissues (Taylor, 1951b). It is thought that this finding may be based on two facts; firstly, organisms present in the mesenteric lymph nodes or for that matter in the intestinal wall must occasionally, by sheer weight of numbers, find their way into the blood stream and thence throughout the system. M. johnei is known to be an organism resistant to destruction within the animal body and in all probability it may remain as a commensal for long periods in many of the tissues and glands. Secondly, the technique of the isolation of M. johnei in culture from tissue has been greatly improved in recent years (Taylor, 1950) and there can be little doubt that the isolation of the organism from these so-called unusual sites is dependant purely upon this improvement, as the bacilli are not normally present therein in large numbers. The exception is provided by the acute septicaemic type of the disease, which is uncommon. It may occur occasionally in animals showing clinical symptoms and is characterised by acute haemorrhagic enteritis with the passage of blood and enormous numbers of organisms in the faeces. At the post-mortem examination there may even be ulceration of the mucous surface of the bowel. From such a case an almost confluent growth of colonies may be obtained in culture from such tissues as the liver and lungs.

Sheep:

The presence of the lesions of Johne's disease in the sheep may pass

pass unnoticed at autopsy unless specific search is made. The existence of at least two varieties of the disease in sheep in this country has been suspected by several authors and recently has been confirmed by the isolation in culture of a strongly pigmented organism which may be presumed to be a variety of the classical bovine type of M. johnei (Taylor, 1951a). The form of the disease caused by the bovine organism has been recognised for many years; the intestine is usually thickened but without marked corrugation and the bacilli, although they may be sparse in stained films of the mucous membrane, are normally present in greater numbers than is usual in the bovine disease. The second form, from which the pigmented variety has been isolated, has so far been recorded only in Scotland, where it is known to be widespread in its distribution (Taylor, 1945). It may be recognised readily at autopsy by the orange colour of the lesions in the intestine, which may show little or no thickening. Films or smears prepared from such lesions show the bacilli to be present in enormous numbers comparable only to those seen in the form of tuberculosis in wood pigeons originally described in this country by McDiarmid (1948).

CONTROL

If it be accepted, and there is no evidence to the contrary, that only young animals can be so infected by Johne's bacilli that they may develop clinical signs of the disease in later life, and also that the organisms are to be found only in the faeces of infected animals, then it would appear essential to rear calves as free as possible from contact with the faeces of their elders. The more obvious methods of control, such as the early detection and removal of clinical cases, the provision of a clean water supply, the fencing of ponds, etc., should not, of course, be abandoned, but good results may sometimes be achieved by removing the

the calf from its dam at an early stage in its career and feeding it by hand. It should be remembered that cows may excrete numbers of organisms with their faeces for many months before clinical symptoms become apparent and that very young calves are the most susceptible to infection. In this connection, it was shown experimentally by Hagan (1938) that calves kept free from infection for the first four months of life but subsequently maintained in an infected herd did not contract the disease.

Vaccination:

The value of vaccination against chronic infections such as are produced by the pathogenic mycobacteria is notoriously difficult to assess, as witness the world-wide controversy, argued for many years, over the use of B.C.G. It is normally impossible to conduct properly controlled experiments on tuberculosis in man, which explains much of the difficulty with B.C.G.; but the veterinary research worker is more fortunate in this respect, in that he may undertake controlled experiments with diseases in their natural hosts, all of which may be artificially infected, tested, slaughtered and properly examined. It is, therefore, perhaps surprising that such work has never been adequately done in the case of vaccination against Johne's disease. Vallee, Rinjard and Vallee in 1934 announced good results following the use of their vaccine upon many thousands of animals in the field but so far as the writer is aware there is little evidence, based upon controlled experimental work, which would enable a true assessment of the vaccine to be made beyond that published by Hagan (1935). He used ten vaccinated and ten control calves and found that although the vaccinated animals became infected they withstood the actual disease better than did the controls. In Iceland, Sigurdsson and Trygvadottir (1949),

(1949), using killed laboratory strains of M. johnei originally isolated from cattle and suspended in liquid paraffin, found that a "powerful and protracted serological response" followed its subcutaneous inoculation into sheep, while in this country, Doyle (1945) has shown that the subcutaneous inoculation of a similar vaccine may be regarded as harmless to cattle. The use of vaccine in this country, however, where tuberculosis is still a common infection among cattle, presents several difficulties, chief of which is the type of reaction given to the comparative tuberculin test by vaccinated animals. Ritchie and Robertson, who are studying this problem in Edinburgh, have produced evidence in a personal communication that the comparative test is incapable of distinguishing between cattle vaccinated against Johne's disease and animals which have an infection with bovine tubercle bacilli superimposed upon vaccination. In other words, vaccination against Johne's disease will mask tuberculous infection as judged by the test. This is a serious difficulty, and in these times, when a real attempt is being made to eradicate bovine tuberculosis, may preclude the use of vaccine except in known tubercle-free areas.

Treatment:

The use of iron, formalin, etc., in the treatment of Johne's disease has been generally discredited, but the idea of therapy has been stimulated recently by the widespread use of antibiotics. Of these, streptomycin is said to be active against M. johnei in vitro and it has been used in the United States (Larsen, Vardaman & Groth, 1950) but without conspicuous success, possibly because of the cost of the drug prohibits its use over an extended period in adequate dosage. Within the last two years several authors (see Francis & Spinks, 1950) have commented favourably upon the therapeutic effect of 4: 4 diaminodiphenylsulphone (DDS) in rat leprosy

leprosy and leprosy in man. The use of sulphone as a therapeutic agent in Johne's disease, which in many ways is similar to these conditions, might therefore be worthy of experimental trial.

DISCUSSION

Of the four so-called major diseases of cattle in this country, tuberculosis is one the eradication of which is but a matter of time and money, while contagious abortion and streptococcal mastitis no longer provide a major problem to agriculture and to the veterinary surgeon. As a result of prolonged research these three diseases may all be diagnosed with comparative ease and certainty and effective remedial measures in one form or another applied.

The control of Johne's disease presents an entirely different problem. Here we are dealing with an organism which is apparently not only capable of living more or less as a commensal in the animal body for long periods, but may also be well able so to conceal its presence that it will give no specific reaction to any test that we may apply. Further, in the majority of cases it produces no clinical sign of its presence. Why some infected animals should become clinical cases of the disease is quite unknown. It is well recognised that symptoms often first appear shortly after calving or any other circumstance which puts a strain upon the normal metabolism of the animal and it has also been suggested that dietetic deficiencies in one form or another may be responsible. In this connection it must be remembered, however, that Johne's disease is an infectious disease and that there are many such, caused by specific agents - either of apparently low virulence or of the nature of commensals - all of which require some external stimulus or "trigger mechanism" to set them to work to cause clinical signs of disease.

As to the incidence of the clinical disease among the cattle of this country, there is no accurate information. Twort and Ingram (1913) believed it to be "prevalent all over England," an observation more recently confirmed by Doyle and Spears (1951). Admittedly a reasonably accurate knowledge of the incidence is not easily acquired; many owners of stock do not advertise its presence and in numerous instances the veterinary surgeon is not consulted; the farmer knows the disease to be incurable in the present state of our knowledge and disposes of the animal in one way or another. The fact remains that a knowledge of the relative importance of the disease in the country to-day would be of value in determining the effort that may be required in order to control it.

As a problem for serious research the study of Johne's disease is unproductive of quick results and the technical difficulties involved are very great. Considerable work is yet required upon the improvement of laboratory techniques, diagnosis by allergic or serological methods, the efficiency of vaccination and upon treatment of the infection by therapeutic measures if we are not to remain remarkably helpless either to diagnose or control Johne's disease.

SUMMARY

It is now known that a considerable percentage of what can be regarded as healthy cattle may in fact be infected with Mycobacterium johnei, but it is extremely unlikely that more than a small proportion of these ever become clinical cases of the disease. Diagnosis of the pre-clinical case is extremely difficult if not impossible in the majority of animals as there is no specific allergic or serological test which is now regarded as reliable.

Improvements in the technique of the isolation of M. johnei in culture

culture have shown that the organism regularly may be recovered post-mortem from clinical cases, not only from the intestine and its associated glands but also from a number of other tissues where it probably exists as a commensal.

In sheep at least two types of Johne's disease are known to exist in Great Britain, one caused by the classical bovine organism and another caused by an organism formerly regarded as uncultivable but now known to be a pigmented variant of M. johnei.

Control of Johne's disease, in the absence of a specific diagnostic method, must be based on the prevention of infection of young stock; a great deal of experimental work is yet required on the possibilities of control by vaccination and therapy.

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Observations on the blood picture of Johne's disease in sheep and cattle with special reference to the magnesium content of the blood.

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On routine examination of blood samples from cases of Johne's disease in sheep it was observed that both the calcium and magnesium contents of the blood were usually lower than the normal. Little has been recorded in the literature of variations in the mineral composition of the blood during infections or debilitating diseases. Stewart and Holman (1944) have shown that in the "pining" disease known as "Cobalt-deficiency" and "Solway Pine," despite a considerable amount of emaciation there is little change in the mineral composition of the blood. Watchorn (1925) reported that in nearly all pathological conditions in man investigated by her, the serum magnesium was found to be increased. She also noted that there was no definite parallelism between calcium and magnesium. Various writers, Brockbank (1926), Halverson, Mohler and Bergeim (1917), amongst others, have shown that in tuberculosis the serum calcium values are on the average slightly lower than normal. They did not record magnesium values.

In the light of our preliminary observations, further cases of Johne's disease in sheep and cattle were examined. In all, blood samples were obtained from 19 sheep and nine cows. One of us (Taylor, 1945) has shown that Johne's disease in sheep may be caused by one of two closely related organisms and the majority of the sheep used in the present work was infected with the ovine type of Myco. johnei. On arrival at the Institute each ewe was diagnosed as a probable case of Johne's disease from clinical symptoms and a faeces examination for acid-alcohol fast organisms; confirmation was subsequently obtained on post-mortem examination. Of the cows, cases one to seven were diagnosed as Johne's disease as a result of clinical examination by veterinary practitioners; cases eight and nine were diagnosed from clinical symptoms

symptoms and faeces examination. Blood samples from six cows (10 to 15) have been added to the experiment. These cows reacted to johnin but showed no clinical symptoms of the disease.

The results of the chemical analysis of the blood samples are recorded in the table.

Examination of the data shows that the sugar, non-protein-nitrogen and chloride content of the blood was, with few exceptions, within the normal range. The one or two values outside the normal range may have been due to excessive emaciation, since a very debilitated animal has usually a low blood sugar and an abnormal blood chloride. In most cases the haemoglobin content of the blood was very low, which again may show that there is a certain degree of anaemia brought about by emaciation similar to that seen in sheep heavily infested with helminths. The greatest variation from the normal is observed in the calcium and magnesium values. With the exception of No. 12, all the ewes had a low blood calcium and, with the exception of Nos. 5 and 16, a very low blood magnesium. The variation from the normal was usually much greater in the case of magnesium than of calcium. The same was observed in Johne's disease in cows in which the blood magnesium was very much lower than normal, although the blood calcium was usually lower than normal or at the lowest range of normality. There is no parallelism between the low haemoglobin, calcium and magnesium contents of the blood since there may be an exceptionally low magnesium with a normal haemoglobin (sheep No.1) or a normal magnesium with a low haemoglobin and calcium (sheep No.5). In the cows, also, there was no strict correlation between the variations of the haemoglobin, calcium and magnesium values from the normal. It will be observed in cows Nos. 10 to 15 which reacted to johnin without showing clinical symptoms of Johne's

ANALYSIS OF BLOOD SAMPLES—VALUES PER 100 ML. BLOOD

Animal	Sugar mg.	Hb. g.	NaCl mg.	Ca mg.	Inorg. P. mg.	Total P. mg.	Mg. mg.	N.P.N. mg.	Amino N mg.
SHEEP									
1	45·6	11·0	550·0	7·60	4·32	10·28	1·62	39·2	5·6
2	34·5	6·4	590·0	8·26	5·50	9·62	1·39	33·7	4·8
3	23·8	5·4	580·0	6·26	4·90	9·25	1·49	28·6	4·9
4	55·8	6·3	580·0	8·53	2·76	8·00	2·08	28·6	5·8
5	40·6	7·3	550·0	7·86	3·84	7·94	2·77	—	6·9
6	38·6	9·3	510·0	7·00	5·44	10·80	2·32	28·2	6·8
7	29·8	9·9	480·0	8·10	4·14	9·00	1·60	28·6	7·4
8	47·2	8·1	520·0	8·60	4·32	9·30	1·60	43·5	7·2
9	68·5	5·9	560·0	7·80	3·83	6·54	2·07	33·2	5·5
10	57·2	8·0	560·0	7·70	4·32	8·32	1·67	21·8	5·7
11	—	—	—	8·40	—	—	1·84	—	—
12	75·9	9·2	580·0	10·60	—	—	2·08	—	—
13	52·1	10·7	580·0	8·80	5·06	10·60	2·13	30·0	4·2
14	41·3	8·9	580·0	8·00	5·90	9·20	1·95	35·3	4·7
15	52·9	5·6	530·0	6·80	4·19	7·40	2·12	37·7	3·5
16	44·4	5·5	550·0	8·00	3·86	6·10	2·50	28·7	3·5
17	45·8	9·1	540·0	8·50	3·86	7·70	2·30	35·9	3·7
18	54·3	10·7	510·0	7·30	4·52	9·80	2·06	32·4	4·0
19	58·1	7·7	540·0	8·40	4·52	8·30	1·98	38·5	4·2
Normal for Sheep	61·0 ±10·8	11·2 ±1·1	507·0 ±20·7	10·7 ±0·65	5·3 ±0·52	9·9 ±0·94	2·65 ±0·21	33·3 ±5·3	7·3 ±0·47
COWS									
1	57·1	10·2	500·0	8·26	6·60	13·08	1·90	40·8	6·1
2	60·6	6·9	530·0	12·00	5·10	10·36	1·72	30·0	6·5
3	105·3	11·8	540·0	7·50	8·16	16·25	1·98	54·1	9·4
4	77·5	8·2	500·0	8·30	5·68	10·03	1·98	33·3	6·6
5	45·2	8·3	500·0	9·30	9·70	26·10	1·72	30·3	7·1
6	57·8	7·3	500·0	8·80	6·60	11·50	2·28	36·4	5·2
7	75·7	7·6	520·0	9·30	4·00	6·90	2·38	34·3	5·7
8	—	—	—	8·40	—	—	2·17	—	—
9	—	—	—	9·50	—	—	1·52	—	—
10	48·8	9·0	530·0	9·10	4·90	7·60	2·51	34·9	6·3
11	64·5	7·3	510·0	8·70	4·90	6·80	2·61	31·6	6·4
12	52·3	9·8	470·0	9·70	5·20	8·30	2·50	33·7	6·5
13	55·5	8·8	500·0	9·50	4·70	6·70	2·42	28·2	6·1
14	53·2	8·6	490·0	9·70	4·10	5·80	2·75	27·3	6·4
15	46·5	7·9	490·0	9·10	4·00	5·80	2·47	26·4	5·6
Normal for Cows	60·0 ±10·9	9·5 ±1·4	504·0 ±34·4	10·0 ±·72	4·24 ±0·87	7·7 ±1·20	2·98 ±0·28	38·9 ±7·0	4·6 ±0·20

Johne's disease, that, although the haemoglobin was slightly low in some cases, the blood calcium and blood magnesium were normal. Despite the general lowering of the calcium and magnesium, the phosphate content of the blood was usually normal although one or two cases had an exceptionally high blood phosphate.

DISCUSSION

As has been pointed out above, there is usually a very low haemoglobin content of the blood with occasionally a slightly low calcium content in severe debility such as that caused by heavy infestation of helminths. Recently Saifi and Vaughan (1944) have shown that infections also interfere with haemoglobin formation. There is no reference in the literature to any lowering of the magnesium content of the blood in infections or debility. In "cobalt-deficiency" and "Solway pine," debilitating conditions in lambs, Stewart and Holman (1944) have reported that the blood haemoglobin and the blood calcium are within the normal range whilst Stewart (unpublished observations) has shown the magnesium content of the blood in these two diseases to be 3.10 mg. $\pm$ 0.27 and 2.99 mg. $\pm$0.30 per 100 ml. respectively. It would appear, therefore, that the process of emaciation as such need not bring about a lowering of the magnesium content of the blood. In Johne's disease the cause of the condition may be due to some specific action of the bacteria. Future work may throw some light on this subject. From our present knowledge of blood pictures in various pathological conditions the lowering of the magnesium content of the blood in animals showing clinical symptoms of the disease may to some extent assist diagnosis since other useful aids such as examination of faeces for acid-alcohol fast organisms and testing

testing with avian tuberculin or johnin have their limitations.

SUMMARY

It is shown that in sheep and cows exhibiting clinical symptoms of Johne's disease, the haemoglobin and calcium contents of the blood may be low and that the magnesium content may be very low.

It is suggested that this lowering of the magnesium content of the blood may be a useful aid to the diagnosis of the condition.

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The Filtration of *Mycobacterium tuberculosis* and *Mycobacterium stercusis* through gradocol membranes

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The possibility of the existence of a form of Mycobacterium tuberculosis capable of passing the pores of bacteria-proof filters and of giving rise to disease on inoculation into susceptible laboratory animals has been a source of controversy since the original work of Fontes (1910, quoted by Topley and Wilson, 1936, p. 292) was supported by that of Vaudremer (1923), but in the literature we have found only one reference to the use of filters of known pore size. Lewis, Ruckman and James (1934 - 35), using a negative pressure of 20 cm. of mercury and a collodion membrane described by Krueger and Ritter (1930) with a pore diameter of 0.4 μ, showed that the filtrate did not contain particles capable of producing (1) a state of allergy to tuberculin, (2) anatomical evidence of tuberculous infection in experimental animals or (3) growth of tubercle bacilli in appropriate media. They concluded therefore that the cultures tested did not develop filterable forms smaller than 0.4 μ in diameter.

This paper records a series of experiments on the filtration of Mycobacterium stercusis (mist bacillus of Moeller) and of Mycobacterium tuberculosis (human and bovine types). Suspensions of these organisms were drawn through a series of collodion membranes of decreasing pore size and the presence or absence of viable elements in the filtrates was determined by cultural or biological methods. No evidence was obtained which might have suggested the existence of a form of these organisms in any way analogous to an ultravisible virus.

METHODS

Materials from which filtrates were prepared

1. Myco. stercusis 77, no. 3820 of the National Collection of Type Cultures.
2. Two strains of Myco. tuberculosis (bovine type), nos. F 12 and 674 U, and one human strain M, recently isolated from bovine lymph nodes and human sputum respectively.

3. The lungs and spleen of rabbits dying from experimental tuberculosis induced by the inoculation of three recently isolated bovine strains nos. 674 U, 3 and W 68.

4. Tuberculous lymph nodes and lungs from naturally infected cattle.

5. Sputum from advanced cases of pulmonary tuberculosis in man.

6. A strain of louping-ill virus no. 1, in experimental use at this Institute, was used to control the technique and to ensure that the filters had not become choked.

The preparation of suspensions for filtration

Suspension of cultures: 1. Myco. stercusis. Cultures in a medium of minced chick embryo in Tyrode's solution (Soltys, 1942) of 3, 6, 12 and 24 hours' and 3 days' incubation were mixed in equal parts and diluted 1:50, 1:100 and 1:1000 in Tyrode's solution. Cultures of similar age on glycerol egg were washed off by the addition of 5.0 c.c. of Tyrode's solution to each slope and the resulting suspensions mixed as before and diluted 1:10,000.

2. Myco tuberculosis: Equal parts of cultures of various ages on plain serum or on Dorset's egg medium were again mixed in Tyrode's solution, but in this case the organisms had been grown for 24 and 48 hours and 7, 14, 21, 28 and 35 days.

Suspension of infected tissues: The material was first cut as fine as possible with scissors, then ground in a mortar with sand. Tyrode's solution was added so as to give a 20 per cent. suspension of tissue.

Suspension of sputum: The sputa were shown by microscopical and cultural methods to be rich in human tubercle bacilli. One part of sputum was thoroughly shaken with four parts of Tyrode's solution. The mixture was then filtered at once or after autolysis at 37°C. for 12 hours or 3 days.

The preparation of louping-ill virus for filtration: The virus was grown for 4 days at 37°C. in a medium of minced chick embryo in Tyrode's solution which was then centrifuged for 15 minutes at 2000 r.p.m. to remove gross particles.

Filtration

The same technique was used in each experiment. The bacillary suspensions were drawn first through paper pulp and then through Kieselguhr of approximately 1 mm. depth with a negative pressure of 15 in. of mercury. The louping-ill virus was filtered through Kieselguhr only. Equal volumes of the two filtrates were mixed and drawn through gradocol membranes of progressively smaller pore diameter. These were obtained from St. Mary's Hospital, London, and were of the following sizes:- (1) 2.02 or 1.95 μ (2) 1.5 or 1.43 μ (3) 1.00 μ and (4) 0.7 or 0.69 μ. A negative pressure of 10-15 in. of mercury was used and filtration was continued for half to one minute. The actual procedure was that 40 c.c. of the mixed virus and bacterial suspension were drawn through the membrane of greatest pore size, 10 c.c. of the filtrate being retained for examination, leaving 30 c.c. to be passed through the next size and so on. Thus only 10 c.c. remained to be drawn through the membrane of smallest pore size.

The examination of filtrates for viable elements

Myco. stercusis in filtrates was demonstrated culturally, Myco. tuberculosis by the inoculation of guinea-pigs, and louping-ill virus by the intracerebral inoculation of mice. In experiments with Myco. stercusis 0.25 c.c. of each filtrate was spread over each of ten tubes of glycerol egg medium and 1.0 c.c. was added to a 25 c.c. conical flask of chick embryo medium. Cultures apparently negative after 7 days' incubation were subcultivated on the same media.

In every experiment with Myco. tuberculosis the filtrate from each membrane was inoculated into two guinea-pigs of 400-500 g. weight. One received 3-5 c.c. intraperitoneally, the other 3-5 c.c. subcutaneously.

In the experiments with louping-ill virus three mice were inoculated

inoculated intracranially with 0.1 c.c. of each filtrate, or, in some cases only, of that which had passed the membranes of smaller pore size. Three mice were similarly inoculated with unfiltered material.

Examination of guinea-pigs

An intradermal tuberculin test was performed on each guinea-pig 3, 6 and 12 weeks after inoculation, using 0.1 c.c. of a 1:5 dilution of Weybridge mammalian tuberculin (Ministry of Agriculture & Fisheries). Animals which gave a positive reaction were destroyed after the first or second test, while those giving a consistently negative reaction were killed after the third test. An autopsy was performed on every animal and a careful search made for tuberculous lesions. If these were found, the diagnosis was confirmed microscopically and culturally. When no lesions were found, the inguinal, mesenteric and hepatic lymph nodes were removed, ground in a mortar with saline and centrifuged. The deposit was examined microscopically and culturally and inoculated into one guinea-pig. This animal was tested with tuberculin as before and killed six weeks after inoculation.

RESULTS

The results obtained are shown in tables I-V. In all the experiments Myco. stercusis and the human tubercle bacillus were retained by a membrane of A.P.D. 1.0 μ while the bovine tubercle bacillus, when not retained by a similar membrane, was unable to penetrate one of A.P.D. 0.69 or 0.7 μ. It is commonly held that the bovine organism tends on the average to be smaller than the human type, although this tendency is said to be largely if not entirely dependent upon environmental factors. The number of experiments in this series is too small to permit of a definite conclusion being drawn, but on no occasion was the human type of organism demonstrable in filtrates

TABLE I

Experiments with Mycobacterium stercusis

Material used in preparation of filtrate	Pore size of membrane in μ	Control of filter by louping-ill virus *	Growth on glycerol egg	Growth on chick embryo medium	Subcultures
Culture of *Myco. stercusis* in chick embryo medium. Diluted 1 : 50	1·95	+	+	+	...
	1·50	+	+	+	...
	1·03	+	—	—	—
	0·70	+	—	—	—
As above but diluted 1 : 100	1·95	...	+	+	...
	1·50	...	+	+	...
	1·03	+	—	—	—
	0·70	+	—	—	—
As above but diluted 1 : 1000	1·95	...	+	+	...
	1·50	...	+	+	...
	1·03	+	—	—	—
	0·70	+	—	—	—
Culture of *Myco. stercusis* on glycerine egg. Diluted 1 : 10,000	1·95	...	+	+	...
	1·50	...	+	+	...
	1·03	+	—	—	—
	0·70	+	—	—	—

* + = Mice inoculated intracerebrally developed typical louping-ill.

TABLE II

Experiments with cultures of Mycobacterium tuberculosis

Material used in preparation of filtrate	Pore size of membrane in μ	Route of inoculation	Tuberculin tests I	Tuberculin tests II	Tuberculin tests III	Duration of life in days	Results
Cultures of bovine strain 674 U on plain serum	2·02	IP.	+	+	...	55	G.T.B.
		SC.	+	+	...	55	G.T.B.
	1·43	IP.	−	−	−	84	−
		SC.	+	+	...	55	G.T.B.
	1·00	IP.	−	−	−	84	−
		SC.	+	+	...	55	G.T.B.
	0·70	SC.	−	−	−	84	−
		IP.	−	−	−	84	−
Cultures of bovine strain F 12 on plain egg	2·02	IP.	+	+	...	47	G.T.B.
		SC.	+	+	...	47	G.T.B.
	1·43	IP.	+	+	...	47	G.T.B.
		SC.	+	+	...	47	G.T.B.
	1·00	IP.	−	−	−	81	−
		SC.	−	−	−	81	−
	0·70	IP.	−	−	−	81	−
		SC.	−	−	−	81	−
Cultures of human strain Mac on glycerol egg	1·95	IP.	+	+	...	44	T.B.
		SC.	+	+	...	44	T.B.
	1·50	IP.	+	+	...	44	T.B.
		SC.	+	+	...	44	T.B.
	1·00	IP.	−	−	−	89	−
		SC.	−	−	−	89	−
	0·69	IP.	−	−	−	89	−
		SC.	−	−	−	89	−

IP. = Intraperitoneally
SC. = Subcutaneously
G.T.B. = Generalised tuberculosis
T.B. = Tuberculosis, not generalised

TABLE III

Experiments with tuberculous tissues from rabbits infected with bovine strains

Material used in preparation of filtrate	Pore size of membrane in μ	Route of inoculation	Tuberculin tests			Duration of life in days	Results
			I	II	III		
Lung and spleen of infected rabbit (strain F 12)	2·02	IP.	+	...	...	26	G.T.B.
		SC.	+	+	...	56	G.T.B.
	1·43	IP.	+	...	...	26	G.T.B.
		SC.	+	+	...	56	G.T.B.
	1·00	IP.	+	...	...	26	G.T.B.
		SC.	+	+	...	56	G.T.B.
	0·70	IP.	−	−	...	56	−
		SC.	−	−	−	89	−
Lung and spleen of infected rabbit (strain 3)	2·02	IP.	+	+	...	49	G.T.B.
		SC.	+	+	...	49	G.T.B.
	1·43	IP.	+	+	...	49	G.T.B.
		SC.	+	+	...	49	G.T.B.
	1·00	IP.	−	−	−	105	−
		SC.	−	−	−	105	−
	0·70	IP.	−	−	−	105	−
		SC.	−	−	−	105	−
Lung and spleen of infected rabbit (strain W 68)	2·02	IP.	+	+	...	46	G.T.B.
		SC.	+	+	...	46	G.T.B.
	1·43	IP.	+	+	...	46	G.T.B.
		SC.	+	+	...	46	G.T.B.
	1·00	IP.	+	+	...	46	G.T.B.
		SC.	+	+	...	46	G.T.B.
	0·70	IP.	−	−	−	98	−
		SC.	−	−	−	98	−

TABLE IV

Experiments with tuberculous bovine tissues

Material used in preparation of filtrate	Pore size of membrane in μ	Route of inoculation	Tuberculin tests			Duration of life in days	Results
			I	II	III		
Tuberculous bovine bronchial lymph node and lung	1·95	IP.	+	+	...	45	G.T.B.
		SC.	+	+	...	45	G.T.B.
	1·43	IP.	+	+	...	45	G.T.B.
		SC.	+	+	...	45	G.T.B.
	1·00	IP.	+	+	...	45	G.T.B.
		SC.	+	+	...	45	G.T.B.
	0·70	IP.	—	—	—	92	—
		SC.	—	—	—	92	—
Tuberculous bovine bronchial lymph node	1·95	IP.	+	+	...	43	G.T.B.
		SC.	+	+	...	43	G.T.B.
	1·50	IP.	+	+	...	43	G.T.B.
		SC.	+	+	...	43	G.T.B.
	1·00	IP.	+	+	...	43	G.T.B.
		SC.	+	+	...	43	G.T.B.
	0·69	IP.	—	—	—	85	—
		SC.	—	—	—	85	—
Tuberculous bovine retro-pharyngeal lymph node	1·95	IP.	+	+	...	44	T.B.
		SC.	+	+	...	44	T.B.
	1·50	IP.	+	+	...	44	G.T.B.
		SC.	+	+	...	44	T.B.
	1·00	IP.	—	—	—	90	—
		SC.	—	—	—	90	—
	0·69	IP.	—	—	—	90	—
		SC.	—	—	—	90	—

TABLE V

Experiments with tuberculous sputum

Material used in preparation of filtrate	Pore size of membrane in μ	Route of inoculation	Tuberculin tests I	II	III	Duration of life in days	Results
Fresh sputum . . .	1·95	IP.	+	+	...	46	G.T.B.
		SC.	+	+	...	46	G.T.B.
	1·43	IP.	+	+	...	46	G.T.B.
		SC.	+	+	...	46	G.T.B.
	1·00	IP.	−	−	−	80	−
		SC.	−	−	−	80	−
	0·70	IP.	−	−	−	80	−
		SC.	−	−	−	80	−
Sputum autolysed at 37° C. for 12 hours	1·95	IP.	+	+	...	47	T.B.
		SC.	+	+	...	47	T.B.
	1·43	IP.	+	+	...	47	T.B.
		SC.	+	+	...	47	T.B.
	1·00	IP.	−	−	−	79	−
		SC.	−	−	−	79	−
	0·70	IP.	−	−	−	79	−
		SC.	−	−	−	79	−
Sputum autolysed at 37° C. for 3 days	1·95	IP.	−	±	+	87	T.B.
		SC.	−	−	−	87	−
	1·50	IP.	−	−	−	87	−
		SC.	−	±	+	87	T.B.
	1·00	IP.	−	−	−	87	−
		SC.	−	−	−	87	−
	0·69	IP.	−	−	−	87	−
		SC.	−	−	−	87	−

filtrates drawn through membranes of A.P.D. 1.0 μ. Yet when using the bovine bacilli of both animal and cultural origin, on five of eight occasions such a filtrate was infective for guinea-pigs.

In the experiments on the filtration of sputum (table V) the somewhat irregular results obtained with sputum autolysed at 37°C. for 3 days may have been due to the fact that putrefaction was advanced and the material was in a very offensive condition.

The control louping-ill virus was demonstrated in the final filtrate in each experiment.

DISCUSSION

It was shown by Elford (1931) that the great majority of micro-organisms are effectively retained by a collodion membrane filter of A.P.D. 0.75 μ. The known bacteria that will pass such a filter are few in number and include the spirochaetes (Hindle and Elford, 1933), the causal organism of pleuro-pneumonia and the sewage organism isolated by Laidlaw and Elford (1936). The results show that there can be no justification for including Myco. tuberculosis in this group, as it was in every case retained by a membrane of A.P.D. 0.7 μ.

Several workers have described in guinea-pigs inoculated with filtrates of tuberculous material lesions which, while not apparently those of typical tuberculosis, have been described as a modified form of the disease. This phenomenon was probably due, in some cases at least, to the passage of organisms through the filter and the inoculation of a very small number of viable tubercle bacilli, as Schwabacher and Wilson (1936-37) were able to produce atypical forms of tuberculosis in guinea-pigs by the inoculation of less than twenty virulent organisms. On other occasions it may have been due to the fact that "work of this type is beset with possibilities of

of technical error" (Topley and Wilson, 1936, p.292). No such forms of the disease were found in any of the experiments described; the inoculated animals either remained healthy and showed no lesions whatever when examined at autopsy twelve weeks after inoculation, or they developed active tuberculosis. This refutes a possible criticism that the original material was too highly diluted to allow organisms to appear in each filtrate, especially in view of the fact that every membrane permitted the passage of louping-ill virus.

No evidence was found, therefore, of a filterable form of the tubercle bacillus infective for guinea-pigs or in any way analgous to the viruses. Even the very small granular forms of the organism, originally described by Much (1907) and shown by many subsequent workers to be present in considerable quantity in very young cultures, and for which due allowance was made in the preparation of material for filtration, are apparently either unable to penetrate a membrane of A.P.D. 0.7 μ or non-infective for guinea-pigs, an alternative which appears somewhat unlikely.

SUMMARY

1. Experiments are described on the filtration of Mycobacterium stercusis and the human and bovine types of Mycobacterium tuberculosis through gradocol membranes of known pore diameter.

2. Viable elements of each organism capable either of growth on artificial media or of producing severe generalised disease in experimental animals were constantly demonstrated in filtrates drawn through the membranes of average pore diameter 2.0 μ and 1.5 μ. Myco. tuberculosis (bovine type) was also shown to be capable of passing a membrane of A.P.D. 1.0 μ in 5 of 8 instances.

3. The methods employed were unable to detect viable elements in filtrates

filtrates drawn through membranes of A.P.D. 0.7 μ .

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